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Attorney Docket No. 1095R

April 28, 2000

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**UTILITY PATENT APPLICATION TRANSMITTAL**

Inventor(s): Dean Della Penna, Eva Collakova, Sean J. Coughlan, Timothy G. Helentjaris

Title: Phytyl/Prenyltransferase Nucleic Acids, Polypeptides and Uses Thereof

**APPLICATION ELEMENTS**

1. ☐ Fee Transmittal Form (Submit an original, and a duplicate for fee processing)
2. ☒ Specification [Total Pages 70]  
(Preferred arrangement set forth below)
  - Descriptive title of the Invention
  - Cross Reference to Related Applications
  - Statement Regarding Fed sponsored R & D
  - Reference to Microfiche Appendix
  - Background of the Invention
  - Brief Summary of the Invention
  - Brief Description of the Drawings (if filed)
  - Detailed Description
  - Claim(s)
  - Abstract of the Disclosure
3. ☐ Drawing(s) (35 USC 113) [Total Sheets       ]
  - a. ☐ Formal
  - b. ☐ Informal

09/560761-1

4. ☐ Oath or Declaration [Total Pages \_\_\_\_]
- a. ☐ Newly executed (original or copy)
- b. ☐ Copy from a prior application (37 CFR 1.63(d))  
(for continuation/divisional with Box 17 completed)  
[Note Box 5 below]
- i. ☐ DELETION OF INVENTOR(S)  
Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
5. ☐ Incorporation By Reference (useable if Box 4B is checked)  
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
6. ☐ Microfiche Computer Program (Appendix)
7. ☒ Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
- a. ☒ Computer Readable Copy
- b. ☒ Paper Copy (identical to computer copy) [Total Pages 33]
- c. ☒ Statement verifying identity to above copies

**ACCOMPANYING APPLICATION PARTS**

8. ☐ Assignment Papers (cover sheet & document(s))
9. ☐ 37 CFR 3.73(b) Statement ☐ Power of Attorney  
(where there is an assignee)
10. ☐ English Translation Document (if applicable)
11. ☐ Information Disclosure Statement (IDS/PTO-1449) ☐ Copies of IDS Citations
12. ☐ Preliminary Amendment
13. ☒ Return Receipt Postcard (MPEP 503) (Should be specifically itemized)
14. ☐ Small Entity Statement(s) ☐ Statement filed in prior application  
Status still proper and desired

15. ☐ Certified Copy of Priority document(s)

16. ☐ Other:

17. If a **CONTINUING APPLICATION**, check the appropriate box and supply the requisite information: ☐ Continuation ☐ Divisional ☒ Continuation-in-part (CIP) of prior application No: 09/307,460.

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Attorney Docket No.: 1095R

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Respectfully submitted,



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**PHYTYL/PRENYLTRANSFERASE NUCLEIC ACIDS, POLYPEPTIDES**  
**AND USES THEREOF**

**Dean Della Penna**  
**Eva Collakova**  
**Sean J. Coughlan**  
**Timothy G. Helentjaris**

**CROSS-REFERENCE TO RELATED APPLICATIONS**

This application claims benefit of U.S. Patent Application Serial No. 09/307,460 filed May 7, 1999, which is hereby incorporated by reference.

**TECHNICAL FIELD**

The present invention relates generally to plant molecular biology. More specifically, it relates to nucleic acids and methods for modulating their expression in plants.

**BACKGROUND OF THE INVENTION**

The chloroplasts of higher plants contain and elaborate many unique, interconnected biochemical pathways that produce an array of compounds that not only perform vital plastid functions but are also important from agricultural and nutritional perspectives. One class of lipid soluble, chloroplastically synthesized compounds are the prenyllipids, plastoquinone and tocopherols. Plastoquinone is a fundamentally important component of the chloroplast photosynthetic electron transport chain and accounts for up to 50% of the total prenyllipid pool in green tissues. Tocopherols collectively account for up to 40% of the total prenyllipids pool in green plastids and have a well documented role in mammals as an antioxidant [Liebler, 1993] and a similar, though less well understood antioxidant role in plants [Hess, 1993]. The essential nutritional value of tocopherols has been known for over 70 years [Mason, 1980]. Despite the well studied, wide-spread importance of these chloroplastic compounds to human nutrition, agriculture and biochemical processes within plant cells, much remains to be learned at the molecular level about their biosynthesis.

Plastoquinone and tocopherols are the most abundant prenyllipids in the plastid and are synthesized by the common pathway reviewed in Hess, 1993 and Soll, 1987. The head group for both compounds, homogentisic acid, is produced from p-hydroxyphenylpyruvic acid by the enzyme p-hydroxyphenylpyruvic acid dioxygenase in a reaction that catalyzes both an oxidation and decarboxylation. Homogentisic acid is subject to phytylation/prenylation (phytyl and solanyl, C20 and C45, respectively) coupled to a simultaneous decarboxylation to form the first true tocopherol and plastoquinone intermediates, 2-demethyl-phytylplastoquinol and 2-demethylplastoquinol-9, respectively. A single ring methylation occurs on 2-demethylplastoquinol to yield plastoquinol-9 that is then oxidized to plastoquinone-9. The preferred route in spinach for  $\alpha$ -tocopherol formation is thought to be 1) ring methylation of 2-demethylphytylplastoquinol, to yield phytylplastoquinol, 2) cyclization to yield gamma-tocopherol and, finally, 3) a second ring methylation to yield  $\alpha$ -tocopherol. The first ring methylation in both tocopherol and plastoquinone synthesis is thought to be carried out by a single enzyme that is specific for the site of methylation on the ring but has broad substrate specificity and accommodates both classes of compounds. The final methylation enzyme (gamma tocopherol methyl transferase) is the only enzyme of the pathway that has been purified from plants to date (dHarlingue and Camara, 1985). All other enzymatic activities of tocopherol/plastoquinone synthesis have been localized to the inner chloroplast envelope by fractionation studies except p-hydroxyphenylpyruvic acid dioxygenase and the tocopherol cyclase enzyme. Difficulties with cell fractionation methods, low activities for some of the enzymes, substrate stability and availability and assay problems make studying the pathway biochemically extremely challenging.

The fact that tocopherol and plastoquinone levels, ratios and total amounts vary by orders of magnitude in different plant tissues and developmental stages indicates the pathway is both highly regulated and highly flexible and has potential for quantitative and qualitative manipulation. However, while biochemical analysis has been useful in deciphering the biosynthetic pathway such studies have provided almost no insight into how bulk carbon flow through the pathway is regulated or how differing amounts of tocopherols or plastoquinone are synthesized.

## SUMMARY OF THE INVENTION

It is an object of the present invention to provide nucleic acids and polypeptides relating to the biosynthesis of tocopherol and plastiquinone.

It is another object of the present invention to provide nucleic acids and polypeptides that can be used to identify proteins involved in tocopherol and plastiquinone biosynthesis.

It is another object of the present invention to provide antigenic fragments of the polypeptides of the present invention.

It is another object of the present invention to provide transgenic plants comprising the nucleic acids of the present invention.

It is another object of the present invention to provide methods for modulating, in a transgenic plant, the expression of the nucleic acids of the present invention.

It is another object of the present invention to provide a method for modulating the level of tocopherols and plastiquinone in a plant.

Other aspects of the present invention include expression cassettes comprising the nucleic acid operably linked to a promoter, host cells transfected with the expression cassette, and transgenic plants and seeds comprising the expression cassette.

In a further aspect, the present invention relates to a method of modulating expression of the nucleic acids in a plant, comprising the steps of

- (a) transforming a plant cell with an expression cassette comprising a nucleic acid of the present invention operably linked to a promoter in sense or antisense orientation;
- (b) growing the plant cell under plant growing conditions to produce a regenerated plant capable of expressing the nucleic acid for a time sufficient to modulate expression of the nucleic acids in the plant compared to a corresponding non-transformed plant.

Expression of the nucleic acids encoding the proteins of the present invention can be increased or decreased relative to a non-transformed control plant.

## DETAILED DESCRIPTION OF THE INVENTION

Tocopherols are synthesized in the inner plastid membrane. The first committed step in the pathway is the condensation of the homogentisate head group with the phytyl tail catalyzed by an integral membrane protein: homogentisate: phytyl transferase. The present polypeptides catalyze the condensation of homogentisic acid with phytyldiphosphate or geranylgeranyl pyrophosphate to produce the first intermediates in tocopherol or tocotrienol synthesis, respectively.

The phytylation/prenylation of homogentisic acid is a likely key regulatory step for "tail" synthesis and in determining the relative amounts of tocopherols, tocotrienols and plastoquinone produced as it is the branchpoint for the tocopherol and plastoquinone arms of the pathway.

One purpose of this invention is to modulate a prenyllipid biosynthetic pathway, such as the plastoquinone and tocopherol pathways. The modulation of the pathway may be an up regulation or down regulation of the amount or activity of a prenyllipid (ie. plastoquinone or tocopherol), or of an intermediate in a pathway (ie. 2-demethyl-phytylplastoquinol or 2-demethylplastoquinol-9).

## DEFINITIONS

The term "isolated" refers to material, such as a nucleic acid or a protein, which is: (1) substantially or essentially free from components which normally accompany or interact with the material as found in its naturally occurring environment or (2) if the material is in its natural environment, the material has been altered by deliberate human intervention to a composition and/or placed at a locus in the cell other than the locus native to the material.

The terms polypeptide, "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The essential nature of such analogues of naturally occurring amino acids is that, when incorporated into a protein, that protein is specifically reactive to antibodies elicited to the same protein but consisting entirely of naturally occurring amino acids. The terms "polypeptide", "peptide" and "protein" are also inclusive of modifications including, but not limited



to, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation. Further, this invention contemplates the use of both the methionine-containing and the methionine-less amino terminal variants of the protein of the invention.

As used herein, "plant" includes but is not limited to plant cells, plant tissue and plant seeds.

As used herein, "promoter" includes reference to a region of DNA upstream from the start of transcription and involved in recognition and binding of RNA polymerase and other proteins to initiate transcription.

By "fragment" is intended a portion of the nucleotide sequence or a portion of the amino acid sequence and hence protein encoded thereby. Preferably fragments of a nucleotide sequence may encode protein fragments that retain the biological activity of the native nucleic acid. However, fragments of a nucleotide sequence which are useful as hybridization probes generally do not encode fragment proteins retaining biological activity. Fragments of a nucleotide sequence are generally greater than 10 nucleotides, preferably at least 20 nucleotides and up to the entire nucleotide sequence encoding the proteins of the invention. Generally probes are less than 1000 nucleotides and preferably less than 500 nucleotides. Fragments of the invention include antisense sequences used to decrease expression of the inventive nucleic acids. Such antisense fragments may vary in length ranging from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, up to and including the entire coding sequence.

By "variants" is intended substantially similar sequences. Generally, nucleic acid sequence variants of the invention will have at least 50%, 60%, 70%, or preferably 80%, more preferably at least 90% and most preferably at least 95% sequence identity to the native nucleotide sequence.

Generally, polypeptide sequence variants of the invention will have at least about 55%, 60%, 70%, 80%, or preferably at least about 90% and more preferably at least about 95% sequence identity to the native protein.

As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences includes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in

reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g. charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ for conservative substitutions, the percent identity may be adjusted upward to correct for the conservative nature of the substitution. Means for making this adjustment are well known to those skilled in the art, and typically involve scoring a conservative substitution as a partial rather than a full mismatch.

Methods of alignment of sequences for comparison are well-known in the art. Two methods are used herein to define the present invention. The first is the BLAST 2.0 suite of programs using default parameters. Altschul *et al.*, *Nucleic Acids Res.* 25:3389-3402 (1997). Software for performing BLAST analyses is publicly available, e.g., through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). The second is the GAP program, available as part of the Wisconsin Genetics Software Package, that uses the algorithm of Needleman and Wunsch (J. Mol. Biol. 48:443-453, 1970) to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. Default gap creation penalty values and gap extension penalty values in Version 10 of the Wisconsin Genetics Software Package for nucleotide sequences are 50 and 3, respectively, and for protein sequences are 8 and 2, respectively. Unless otherwise specified, references to the GAP program or algorithm refer to the GAP program or algorithm in version 10 of the Wisconsin Genetics Software Package. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or greater. The scoring matrix used in Version 10 of the Wisconsin Genetics Software Package is BLOSUM62 (see Henikoff & Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

When GAP is used to compute % sequence identities for sequences of differing length, results determined by GAP may be reduced for non-overlapping nucleotides or amino acids in the longer sequence. For example, if a sequence of 100 is compared to a sequence of 40, GAP may determine the percent identity to

be 100% if the 40 nucleotides or amino acids of the shorter sequence match 40 nucleotides or amino acids of the larger sequence. This is because GAP may calculate the percent identity based on the total length of the shorter sequence. However, where this specification, including the claims, specifies the sequence identity being computed by GAP, the GAP percentage identity should be re-calculated as a percentage of the longer sequence and any nucleotides or amino acids in the larger sequence that extend beyond the shorter sequence would not count as a match. In the example provided above this would give a percent identity of 40%.

Other methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman, *Adv. Appl. Math.* 2:482 (1981); by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48:443 (1970); by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci.* 85:2444 (1988); by computerized implementations of these algorithms, including, but not limited to: CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, California; GAP, BESTFIT, FASTA, BLAST and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wisconsin, USA; the CLUSTAL program is well described by Higgins and Sharp, *Gene* 73:237-244 (1988); Higgins and Sharp, *CABIOS* 5:151-153 (1989); Corpet *et al.*, *Nucleic Acids Research* 16:10881-90 (1988); Huang *et al.*, *Computer Applications in the Biosciences* 8:155-65 (1992), and Pearson *et al.*, *Methods in Molecular Biology* 24:307-331 (1994).

The BLAST family of programs which can be used for database similarity searches includes: BLASTN for nucleotide query sequences against nucleotide database sequences; BLASTX for nucleotide query sequences against protein database sequences; BLASTP for protein query sequences against protein database sequences; TBLASTN for protein query sequences against nucleotide database sequences; and TBLASTX for nucleotide query sequences against nucleotide database sequences. See *Current Protocols in Molecular Biology*, Chapter 19, Ausubel *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995). Software for performing BLAST analyses is publicly available, e.g., through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). The BLAST algorithm performs a statistical

analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5877 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance.

The term "functional equivalent" means that the sequence of the variant polynucleotide defines a chain that produces a protein having substantially the same biological effect as the protein encoded by the non-variant polynucleotide.

The term "Complement" or "Complementary" when used with respect to a polynucleotide sequence refers to the corresponding base pairs in the same sequence.

The term "Hybridization Probe" refers to a process whereby a polynucleotide is used to find a complementary polynucleotide through the annealing of the two polynucleotides to form a double helix.

The term "Coding Sequence" when used with respect to a complete gene sequence refers to the sequence spanning the start and stop codon, and when used with respect to a partial gene sequence refers to a portion of the coding region spanning the start and stop codon.

## NUCLEIC ACIDS

The isolated nucleic acids of the present invention can be made using (a) standard recombinant methods, (b) synthetic techniques, or combinations thereof. In some embodiments, the polynucleotides of the present invention will be cloned, amplified, or otherwise constructed from a monocot or dicot. In preferred embodiments the monocot is corn, sorghum, barley, wheat, millet, or rice. Preferred dicots include soybeans, sunflower, canola, alfalfa, cotton, potato, cassava, *Arabidopsis thaliana*, tomato, *Brassica* vegetables, peppers, potatoes, apples, spinach, or lettuce.

Functional fragments included in the invention can be obtained using primers that selectively hybridize under stringent conditions. Primers are generally at least 12 bases in length and can be as high as 200 bases, but will generally be from 15 to 75, preferably from 15 to 50. Functional fragments can be identified using a variety of techniques such as restriction analysis, Southern analysis, primer extension analysis, and DNA sequence analysis.

The present invention includes a plurality of polynucleotides that encode for the identical amino acid sequence. The degeneracy of the genetic code allows for such "silent variations" which can be used, for example, to selectively hybridize and detect allelic variants of polynucleotides of the present invention. Additionally, the present invention includes isolated nucleic acids comprising allelic variants. The term "allele" as used herein refers to a related nucleic acid of the same gene.

Variants of nucleic acids included in the invention can be obtained, for example, by oligonucleotide-directed mutagenesis, linker-scanning mutagenesis, mutagenesis using the polymerase chain reaction, and the like. See, for example, Ausubel, pages 8.0.3 - 8.5.9. Also, see generally, McPherson (ed.), *DIRECTED MUTAGENESIS: A Practical approach*, (IRL Press, 1991). Thus, the present invention also encompasses DNA molecules comprising nucleotide sequences that have substantial sequence identity with the inventive sequences.

Variants included in the invention may contain individual substitutions, deletions or additions to the nucleic acid or polypeptide sequences. Such changes will alter, add or delete a single amino acid or a small percentage of amino acids in the encoded sequence. Variants are referred to as "conservatively modified variants" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. When the nucleic acid is prepared or altered synthetically, advantage can be taken of known codon preferences of the intended host.

The present invention also includes "shufflents" produced by sequence shuffling of the inventive polynucleotides to obtain a desired characteristic. Sequence shuffling is described in PCT publication No. 96/19256. See also, Zhang, J. H., *et al.*, *Proc. Natl. Acad. Sci. USA* 94:4504-4509 (1997).

The present invention also includes the use of 5' and/or 3' UTR regions for modulation of translation of heterologous coding sequences. Positive sequence motifs include translational initiation consensus sequences (Kozak, *Nucleic Acids Res.* 15:8125 (1987)) and the 7-methylguanosine cap structure (Drummond *et al.*, *Nucleic Acids Res.* 13:7375 (1985)). Negative elements include stable intramolecular 5' UTR stem-loop structures (Muesing *et al.*, *Cell* 48:691 (1987)) and AUG sequences or short open reading frames preceded by an appropriate AUG in the 5' UTR (Kozak, *supra*, Rao *et al.*, *Mol. and Cell. Biol.* 8:284 (1988)).

Further, the polypeptide-encoding segments of the polynucleotides of the present invention can be modified to alter codon usage. Altered codon usage can be employed to alter translational efficiency and/or to optimize the coding sequence for expression in a desired host or to optimize the codon usage in a heterologous sequence for expression in maize. Codon usage in the coding regions of the polynucleotides of the present invention can be analyzed statistically using commercially available software packages such as "Codon Preference" available from the University of Wisconsin Genetics Computer Group (see Devereaux *et al.*, *Nucleic Acids Res.* 12:387-395 (1984)) or MacVector 4.1 (Eastman Kodak Co., New Haven, Conn.).

For example, the inventive nucleic acids can be optimized for enhanced expression in organisms of interest. See, for example, EPA0359472; WO91/16432; Perlak *et al.*, *Proc. Natl. Acad. Sci. USA* 88:3324-3328 (1991); and Murray *et al.*, *Nucleic Acids Res.* 17:477-498 (1989). In this manner, the genes can be synthesized utilizing species-preferred codons. See, for example, Murray *et al.*, *Nucleic Acids Res.* 17:477-498 (1989), the disclosure of which is incorporated herein by reference.

The present invention provides subsequences comprising isolated nucleic acids containing at least 16 contiguous bases of the inventive sequences. For example the isolated nucleic acid includes those comprising at least 20, 25, 30, 40, 50, 60, 75 or 100 or more contiguous nucleotides of the inventive sequences. Subsequences of the isolated nucleic acid can be used to modulate or detect gene expression by introducing into the subsequences compounds which bind, intercalate, cleave and/or crosslink to nucleic acids.

The nucleic acids of the invention may conveniently comprise a multi-cloning site comprising one or more endonuclease restriction sites inserted into the nucleic acid to aid in isolation of the polynucleotide. Also, translatable sequences may be inserted to aid in the isolation of the translated polynucleotide of the present invention. For example, a hexa-histidine marker sequence provides a convenient means to purify the proteins of the present invention.

A polynucleotide of the present invention can be attached to a vector, adapter, promoter, transit peptide or linker for cloning and/or expression of a polynucleotide of the present invention. Additional sequences may be added to such cloning and/or expression sequences to optimize their function in cloning

The isolated nucleic acid compositions of this invention, such as RNA, cDNA, genomic DNA, or a hybrid thereof, can be obtained from plant biological sources using any number of cloning methodologies known to those of skill in the art. In some embodiments, oligonucleotide probes that selectively hybridize, under stringent conditions, to the polynucleotides of the present invention are used to identify the desired sequence in a cDNA or genomic DNA library.

Exemplary total RNA and mRNA isolation protocols are described in *Plant Molecular Biology: A Laboratory Manual*, Clark, Ed., Springer-Verlag, Berlin (1997); and, *Current Protocols in Molecular Biology*, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995). Total RNA and mRNA isolation kits are commercially available from vendors such as Stratagene (La Jolla, CA), Clontech (Palo Alto, CA), Pharmacia (Piscataway, NJ), and 5'-3' (Paoli, PA). See also, U.S. Patent Nos. 5,614,391; and, 5,459,253.

Typical cDNA synthesis protocols are well known to the skilled artisan and are described in such standard references as: *Plant Molecular Biology: A Laboratory Manual*, Clark, Ed., Springer-Verlag, Berlin (1997); and, *Current Protocols in Molecular Biology*, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995). cDNA synthesis kits are available from a variety of commercial vendors such as Stratagene or Pharmacia.

An exemplary method of constructing a greater than 95% pure full-length cDNA library is described by Carninci *et al.*, *Genomics* 37:327-336 (1996). Other methods for producing full-length libraries are known in the art. See, e.g., Ederly *et al.*, *Mol. Cell Biol.* 15(6):3363-3371 (1995); and, PCT Application WO 96/34981.

It is often convenient to normalize a cDNA library to create a library in which each clone is more equally represented. A number of approaches to normalize cDNA libraries are known in the art. Construction of normalized libraries is described in Ko, *Nucl. Acids. Res.* 18(19):5705-5711 (1990); Patanjali *et al.*, *Proc.*

*Natl. Acad. USA* 88:1943-1947 (1991); U.S. Patents 5,482,685 and 5,637,685; and Soares *et al.*, *Proc. Natl. Acad. Sci. USA* 91:9228-9232 (1994).

Subtracted cDNA libraries are another means to increase the proportion of less abundant cDNA species. See, Foote *et al.* in, *Plant Molecular Biology: A Laboratory Manual*, Clark, Ed., Springer-Verlag, Berlin (1997); Kho and Zarbl, *Technique*, 3(2):58-63 (1991); Sive and St. John, *Nucl. Acids Res.* 16(22):10937 (1988); *Current Protocols in Molecular Biology*, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995); and, Swaroop *et al.*, *Nucl. Acids Res.* 19(8):1954 (1991). cDNA subtraction kits are commercially available. See, e.g., PCR-Select (Clontech).

To construct genomic libraries, large segments of genomic DNA are generated by random fragmentation. Examples of appropriate molecular biological techniques and instructions are found in Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Vols. 1-3 (1989), *Methods in Enzymology*, Vol. 152: *Guide to Molecular Cloning Techniques*, Berger and Kimmel, Eds., San Diego: Academic Press, Inc. (1987), *Current Protocols in Molecular Biology*, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995); *Plant Molecular Biology: A Laboratory Manual*, Clark, Ed., Springer-Verlag, Berlin (1997). Kits for construction of genomic libraries are also commercially available.

The cDNA or genomic library can be screened using a probe based upon the sequence of a nucleic acid of the present invention such as those disclosed herein. Probes may be used to hybridize with genomic DNA or cDNA sequences to isolate homologous genes in the same or different plant species. Those of skill in the art will appreciate that various degrees of stringency of hybridization can be employed in the assay; and either the hybridization or the wash medium can be stringent. The degree of stringency can be controlled by temperature, ionic strength, pH and the presence of a partially denaturing solvent such as formamide.

Typically, stringent hybridization conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long



probes (e.g., greater than 50 nucleotides). Typically the hybridization will be conducted for about 4 to about 12 hours.

Preferably the hybridization is conducted under low stringency conditions which include hybridization with a buffer solution of 30 % formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulfate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50°C. More preferably the hybridization is conducted under moderate stringency conditions which include hybridization in 40 % formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55°C. Most preferably the hybridization is conducted under high stringency conditions which include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60°C.

An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York (1993); and *Current Protocols in Molecular Biology*, Chapter 2, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995). Often, cDNA libraries will be normalized to increase the representation of relatively rare cDNAs.

The nucleic acids of the invention can be amplified from nucleic acid samples using amplification techniques. For instance, polymerase chain reaction (PCR) technology can be used to amplify the sequences of polynucleotides of the present invention and related genes directly from genomic DNA or cDNA libraries. PCR and other *in vitro* amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of the desired mRNA in samples, for nucleic acid sequencing, or for other purposes.

Examples of techniques useful for *in vitro* amplification methods are found in Berger, Sambrook, and Ausubel, as well as Mullis *et al.*, U.S. Patent No. 4,683,202 (1987); and, *PCR Protocols A Guide to Methods and Applications*, Innis *et al.*, Eds., Academic Press Inc., San Diego, CA (1990). Commercially available kits for genomic PCR amplification are known in the art. See, e.g., Advantage-GC Genomic PCR Kit (Clontech). The T4 gene 32 protein (Boehringer Mannheim) can be used to improve yield of long PCR products.

PCR-based screening methods have also been described. Wilfinger *et al.* describe a PCR-based method in which the longest cDNA is identified in the first step so that incomplete clones can be eliminated from study. *BioTechniques*, 22(3): 481-486 (1997).

In one aspect of the invention, nucleic acids can be amplified from a plant nucleic acid library. The nucleic acid library may be a cDNA library, a genomic library, or a library generally constructed from nuclear transcripts at any stage of intron processing. Libraries can be made from a variety of plant tissues.

Alternatively, the sequences of the invention can be used to isolate corresponding sequences in other organisms, particularly other plants, more particularly, other monocots. In this manner, methods such as PCR, hybridization, and the like can be used to identify such sequences having substantial sequence identity to the sequences of the invention. See, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York). and Innis *et al.* (1990), *PCR Protocols: A Guide to Methods and Applications* (Academic Press, New York). Coding sequences isolated based on their sequence identity to the entire inventive coding sequences set forth herein or to fragments thereof are encompassed by the present invention.

The isolated nucleic acids of the present invention can also be prepared by direct chemical synthesis by methods such as the phosphotriester method of Narang *et al.*, *Meth. Enzymol.* 68:90-99 (1979); the phosphodiester method of Brown *et al.*, *Meth. Enzymol.* 68:109-151 (1979); the diethylphosphoramidite method of Beaucage *et al.*, *Tetra. Lett.* 22:1859-1862 (1981); the solid phase phosphoramidite triester method described by Beaucage and Caruthers, *Tetra. Letts.* 22(20):1859-1862 (1981), *e.g.*, using an automated synthesizer, *e.g.*, as described in Needham-VanDevanter *et al.*, *Nucleic Acids Res.*, 12:6159-6168 (1984); and, the solid support method of U.S. Patent No. 4,458,066. Chemical synthesis generally produces a single stranded oligonucleotide. This may be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. One of skill will recognize that while chemical synthesis of DNA is limited to sequences of about 100 bases, longer sequences may be obtained by the ligation of shorter sequences.

## EXPRESSION CASSETTES

In another embodiment expression cassettes comprising isolated nucleic acids of the present invention are provided. An expression cassette will typically comprise a polynucleotide of the present invention operably linked to transcriptional initiation regulatory sequences which will direct the transcription of the polynucleotide in the intended host cell, such as tissues of a transformed plant.

The construction of expression cassettes that can be employed in conjunction with the present invention is well known to those of skill in the art in light of the present disclosure. See, e.g., Sambrook, *et al.*; Molecular Cloning: A Laboratory Manual; Cold Spring Harbor, New York; (1989); Gelvin, *et al.*; Plant Molecular Biology Manual; (1990); Plant Biotechnology: Commercial Prospects and Problems, eds. Prakash, *et al.*; Oxford & IBH Publishing Co.; New Delhi, India; (1993); and Heslot, *et al.*; Molecular Biology and Genetic Engineering of Yeasts; CRC Press, Inc., USA; (1992); each incorporated herein in its entirety by reference.

For example, plant expression vectors may include (1) a cloned plant nucleic acid under the transcriptional control of 5' and 3' regulatory sequences and (2) a dominant selectable marker. Such plant expression vectors may also contain, if desired, a promoter regulatory region (e.g., one conferring inducible, constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific/selective expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

Constitutive, tissue-preferred or inducible promoters can be employed. Examples of constitutive promoters include the cauliflower mosaic virus (CaMV) 35S transcription initiation region, the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumefaciens*, the ubiquitin 1 promoter, the Smas promoter, the cinnamyl alcohol dehydrogenase promoter (U.S. Patent No. 5,683,439), the Nos promoter, the pEmu promoter, the rubisco promoter, the GRP1-8 promoter and other transcription initiation regions from various plant genes known to those of skill.

Examples of inducible promoters are the Adh1 promoter which is inducible by hypoxia or cold stress, the Hsp70 promoter which is inducible by heat stress,

and the PPDK promoter which is inducible by light. Also useful are promoters which are chemically inducible.

Examples of promoters under developmental control include promoters that initiate transcription preferentially in certain tissues, such as leaves, roots, fruit, seeds, or flowers. An exemplary promoter is the anther specific promoter 5126 (U.S. Patent Nos. 5,689,049 and 5,689,051). Examples of seed-preferred promoters include, but are not limited to, 27 kD gamma zein promoter and waxy promote, Boronat,A., Martinez,M.C., Reina,M., Puigdomenech,P. and Palau,J.; Isolation and sequencing of a 28 kD glutelin-2 gene from maize: Common elements in the 5' flanking regions among zein and glutelin genes; Plant Sci. 47, 95-102 (1986) and Reina,M., Ponte,I., Guillen,P., Boronat,A. and Palau,J., Sequence analysis of a genomic clone encoding a Zc2 protein from Zea mays W64 A, Nucleic Acids Res. 18 (21), 6426 (1990). See the following site relating to the waxy promoter: Kloesgen,R.B., Gierl,A., Schwarz-Sommer,ZS. and Saedler,H., Molecular analysis of the waxy locus of Zea mays, Mol. Gen. Genet. 203, 237-244 (1986). Promoters that express in the embryo, pericarp, and endosperm are disclosed in US applications Ser. Nos. 60/097,233 filed August 20, 1998 and 60/098,230 filed August 28, 1998. The disclosures each of these are incorporated herein by reference in their entirety.

Either heterologous or non-heterologous (i.e., endogenous) promoters can be employed to direct expression of the nucleic acids of the present invention. These promoters can also be used, for example, in expression cassettes to drive expression of antisense nucleic acids to reduce, increase, or alter concentration and/or composition of the proteins of the present invention in a desired tissue.

If polypeptide expression is desired, it is generally desirable to include a polyadenylation region at the 3'-end of a polynucleotide coding region. The polyadenylation region can be derived from the natural gene, from a variety of other plant genes, or from T-DNA. The 3' end sequence to be added can be derived from, for example, the nopaline synthase or octopine synthase genes, or alternatively from another plant gene, or less preferably from any other eukaryotic gene.

An intron sequence can be added to the 5' untranslated region or the coding sequence of the partial coding sequence to increase the amount of the mature message that accumulates. See for example Buchman and Berg, *Mol.*

*Cell Biol.* 8:4395-4405 (1988); Callis *et al.*, *Genes Dev.* 1:1183-1200 (1987). Use of maize introns Adh1-S intron 1, 2, and 6, the Bronze-1 intron are known in the art. See generally, *The Maize Handbook*, Chapter 116, Freeling and Walbot, Eds., Springer, New York (1994).

The vector comprising the sequences from a polynucleotide of the present invention will typically comprise a marker gene which confers a selectable phenotype on plant cells. Usually, the selectable marker gene will encode antibiotic or herbicide resistance. Suitable genes include those coding for resistance to the antibiotic spectinomycin or streptomycin (e.g., the *aada* gene), the streptomycin phosphotransferase (SPT) gene coding for streptomycin resistance, the neomycin phosphotransferase (NPTII) gene encoding kanamycin or geneticin resistance, the hygromycin phosphotransferase (HPT) gene coding for hygromycin resistance.

Suitable genes coding for resistance to herbicides include those which act to inhibit the action of acetolactate synthase (ALS), in particular the sulfonylurea-type herbicides (e.g., the acetolactate synthase (ALS) gene containing mutations leading to such resistance in particular the S4 and/or Hra mutations), those which act to inhibit action of glutamine synthase, such as phosphinothricin or basta (e.g., the *bar* gene), or other such genes known in the art. The *bar* gene encodes resistance to the herbicide basta and the ALS gene encodes resistance to the herbicide chlorsulfuron.

Typical vectors useful for expression of nucleic acids in higher plants are well known in the art and include vectors derived from the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* described by Rogers *et al.*, *Meth. In Enzymol.* 153:253-277 (1987). Exemplary *A. tumefaciens* vectors useful herein are plasmids pKYLX6 and pKYLX7 of Schardl *et al.*, *Gene* 61:1-11 (1987) and Berger *et al.*, *Proc. Natl. Acad. Sci. USA* 86:8402-8406 (1989). Another useful vector herein is plasmid pBI101.2 that is available from Clontech Laboratories, Inc. (Palo Alto, CA).

A variety of plant viruses that can be employed as vectors are known in the art and include cauliflower mosaic virus (CaMV), geminivirus, brome mosaic virus, and tobacco mosaic virus.

A polynucleotide of the present invention can be expressed in either sense or anti-sense orientation as desired. In plant cells, it has been shown that

antisense RNA inhibits gene expression by preventing the accumulation of mRNA which encodes the enzyme of interest, see, e.g., Sheehy *et al.*, *Proc. Nat'l. Acad. Sci. USA* 85: 8805-8809 (1988); and Hiatt *et al.*, U.S. Patent No. 4,801,340.

Another method of suppression is sense suppression. Introduction of nucleic acid configured in the sense orientation has been shown to be an effective means by which to block the transcription of target genes. For an example of the use of this method to modulate expression of endogenous genes see, Napoli *et al.*, *The Plant Cell* 2: 279-289 (1990) and U.S. Patent No. 5,034,323.

A method of down-regulation of the protein involves using PEST sequences that provide a target for degradation of the protein.

Catalytic RNA molecules or ribozymes can also be used to inhibit expression of plant genes. The inclusion of ribozyme sequences within antisense RNAs confers RNA-cleaving activity upon them, thereby increasing the activity of the constructs. The design and use of target RNA-specific ribozymes is described in Haseloff *et al.*, *Nature* 334:585-591 (1988).

A variety of cross-linking agents, alkylating agents and radical generating species as pendant groups on polynucleotides of the present invention can be used to bind, label, detect, and/or cleave nucleic acids. For example, Vlassov, V. V., *et al.*, *Nucleic Acids Res* (1986) 14:4065-4076, describe covalent bonding of a single-stranded DNA fragment with alkylating derivatives of nucleotides complementary to target sequences. A report of similar work by the same group is that by Knorre, D. G., *et al.*, *Biochimie* (1985) 67:785-789. Iverson and Dervan also showed sequence-specific cleavage of single-stranded DNA mediated by incorporation of a modified nucleotide which was capable of activating cleavage (*J. Am. Chem. Soc.* (1987) 109:1241-1243). Meyer, R. B., *et al.*, *J. Am. Chem. Soc.* (1989) 111:8517-8519, effect covalent crosslinking to a target nucleotide using an alkylating agent complementary to the single-stranded target nucleotide sequence. A photoactivated crosslinking to single-stranded oligonucleotides mediated by psoralen was disclosed by Lee, B. L., *et al.*, *Biochemistry* (1988) 27:3197-3203. Use of crosslinking in triple-helix forming probes was also disclosed by Home, *et al.*, *J. Am. Chem. Soc.* (1990) 112:2435-2437. Use of N4, N4-ethanocytosine as an alkylating agent to crosslink to single-stranded oligonucleotides has also been described by Webb and Matteucci, *J. Am. Chem. Soc.* (1986) 108:2764-2765; *Nucleic Acids Res.* (1986) 14:7661-7674; Feteritz *et*

al., *J. Am. Chem. Soc.* 113:4000 (1991). Various compounds to bind, detect, label, and/or cleave nucleic acids are known in the art. See, for example, U.S. Patent Nos. 5,543,507; 5,672,593; 5,484,908; 5,256,648; and, 5,681,941.

## PROTEINS

Proteins of the present invention include proteins derived from the native protein by deletion (so-called truncation), addition or substitution of one or more amino acids at one or more sites in the native protein. Such variants may result from, for example, genetic polymorphism or from human manipulation. Methods for such manipulations are generally known in the art.

For example, amino acid sequence variants of the polypeptide can be prepared by mutations in the cloned DNA sequence encoding the native protein of interest. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York); Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods Enzymol.* 154:367-382; Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, New York); U.S. Patent No. 4,873,192; and the references cited therein; herein incorporated by reference. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferred.

In constructing variants of the proteins of interest, modifications to the nucleotide sequences encoding the variants will be made such that variants continue to possess the desired activity. Obviously, any mutations made in the DNA encoding the variant protein must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See EP Patent Application Publication No. 75,444.

The isolated proteins of the present invention include a polypeptide comprising at least 23 contiguous amino acids encoded by any one of the nucleic acids of the present invention, or polypeptides which are conservatively modified variants thereof. The proteins of the present invention or variants thereof can

comprise any number of contiguous amino acid residues from a polypeptide of the present invention, wherein that number is selected from the group of integers consisting of from 23 to the number of residues in a full-length polypeptide of the present invention. Optionally, this subsequence of contiguous amino acids is at least 25, 30, 35, or 40 amino acids in length, often at least 50, 60, 70, 80, or 90 amino acids in length.

The present invention includes catalytically active polypeptides (i.e., enzymes). Catalytically active polypeptides will generally have a specific activity of at least 20%, 30%, or 40%, and preferably at least 50%, 60%, or 70%, and most preferably at least 80%, 90%, or 95% that of the native (non-synthetic), endogenous polypeptide. Further, the substrate specificity ( $k_{cat}/K_m$ ) is optionally substantially similar to the native (non-synthetic), endogenous polypeptide. Typically, the  $K_m$  will be at least 30%, 40%, or 50%, that of the native (non-synthetic), endogenous polypeptide; and more preferably at least 60%, 70%, 80%, or 90%. Methods of assaying and quantifying measures of enzymatic activity and substrate specificity ( $k_{cat}/K_m$ ), are well known to those of skill in the art.

The present invention includes modifications that can be made to an inventive protein without diminishing its biological activity. Some modifications may be made to facilitate the cloning, expression, or incorporation of the targeting molecule into a fusion protein. Such modifications are well known to those of skill in the art and include, for example, a methionine added at the amino terminus to provide an initiation site, or additional amino acids (e.g., poly His) placed on either terminus to create conveniently located restriction sites or termination codons or purification sequences.

A protein of the present invention can be expressed in a recombinantly engineered cell such as bacteria, yeast, insect, mammalian, or preferably plant cells. The cells produce the protein in a non-natural condition (e.g., in quantity, composition, location, and/or time), because they have been genetically altered through human intervention to do so.

Typically, an intermediate host cell will be used in the practice of this invention to increase the copy number of the cloning vector. With an increased copy number, the vector containing the nucleic acid of interest can be isolated in significant quantities for introduction into the desired plant cells.



Host cells that can be used in the practice of this invention include prokaryotes, including bacterial hosts such as *Eschericia coli*, *Salmonella typhimurium*, and *Serratia marcescens*. Eukaryotic hosts such as yeast or filamentous fungi may also be used in this invention. It preferred to use plant promoters that do not cause expression of the polypeptide in bacteria.

Commonly used prokaryotic control sequences include promoters such as the beta lactamase (penicillinase) and lactose (lac) promoter systems (Chang *et al.*, Nature 198:1056 (1977)), the tryptophan (trp) promoter system (Goeddel *et al.*, Nucleic Acids Res. 8:4057 (1980)) and the lambda derived P L promoter and N-gene ribosome binding site (Shimatake *et al.*, Nature 292:128 (1981)). The inclusion of selection markers in DNA vectors transfected in *E. coli* is also useful. Examples of such markers include genes specifying resistance to ampicillin, tetracycline, or chloramphenicol.

The vector is selected to allow introduction into the appropriate host cell. Bacterial vectors are typically of plasmid or phage origin. Expression systems for expressing a protein of the present invention are available using *Bacillus sp.* and *Salmonella* (Palva, *et al.*, Gene 22:229-235 (1983); Mosbach, *et al.*, Nature 302:543-545 (1983)).

Synthesis of heterologous proteins in yeast is well known. See Sherman, F., *et al.*, *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory (1982). Two widely utilized yeast for production of eukaryotic proteins are *Saccharomyces cerevisiae* and *Pichia pastoris*. Vectors, strains, and protocols for expression in *Saccharomyces* and *Pichia* are known in the art and available from commercial suppliers (e.g., Invitrogen). Suitable vectors usually have expression control sequences, such as promoters, including 3-phosphoglycerate kinase or alcohol oxidase, and an origin of replication, termination sequences and the like as desired.

A protein of the present invention, once expressed, can be isolated from yeast by lysing the cells and applying standard protein isolation techniques to the lysates. The monitoring of the purification process can be accomplished by using Western blot techniques or radioimmunoassay of other standard immunoassay techniques.

The proteins of the present invention can also be constructed using non-cellular synthetic methods. Solid phase synthesis of proteins of less than about 50

amino acids in length may be accomplished by attaching the C-terminal amino acid of the sequence to an insoluble support followed by sequential addition of the remaining amino acids in the sequence. Techniques for solid phase synthesis are described by Barany and Merrifield, *Solid-Phase Peptide Synthesis*, pp. 3-284 in *The Peptides: Analysis, Synthesis, Biology. Vol. 2: Special Methods in Peptide Synthesis, Part A.*; Merrifield, *et al.*, *J. Am. Chem. Soc.* 85:2149-2156 (1963), and Stewart *et al.*, *Solid Phase Peptide Synthesis, 2nd ed.*, Pierce Chem. Co., Rockford, Ill. (1984). Proteins of greater length may be synthesized by condensation of the amino and carboxy termini of shorter fragments. Methods of forming peptide bonds by activation of a carboxy terminal end (e.g., by the use of the coupling reagent N,N'-dicyclohexylcarbodiimide) is known to those of skill.

The proteins of this invention may be purified to substantial purity by standard techniques well known in the art, including detergent solubilization, selective precipitation with such substances as ammonium sulfate, column chromatography, immunopurification methods, and others. See, for instance, R. Scopes, *Protein Purification: Principles and Practice*, Springer-Verlag: New York (1982); Deutscher, *Guide to Protein Purification*, Academic Press (1990). For example, antibodies may be raised to the proteins as described herein. Purification from *E. coli* can be achieved following procedures described in U.S. Patent No. 4,511,503. Detection of the expressed protein is achieved by methods known in the art and include, for example, radioimmunoassays, Western blotting techniques or immunoprecipitation.

The present invention further provides a method for modulating (i.e., increasing or decreasing) the concentration or composition of the polypeptides of the present invention in a plant or part thereof. Modulation of the polypeptides can be effected by increasing or decreasing the concentration and/or the composition of the polypeptides in a plant. The method comprises transforming a plant cell with an expression cassette comprising a polynucleotide of the present invention to obtain a transformed plant cell, growing the transformed plant cell under plant forming conditions, and expressing the polynucleotide in the plant for a time sufficient to modulate concentration and/or composition of the polypeptides in the plant or plant part.

In some embodiments, the content and/or composition of polypeptides of the present invention in a plant may be modulated by altering, *in vivo* or *in vitro*,

the promoter of a non-isolated gene of the present invention to up- or down-regulate gene expression. In some embodiments, the coding regions of native genes of the present invention can be altered via substitution, addition, insertion, or deletion to decrease activity of the encoded enzyme. See, e.g., Kmiec, U.S. Patent 5,565,350; Zarling *et al.*, PCT/US93/03868.

In some embodiments, an isolated nucleic acid (e.g., a vector) comprising a promoter sequence is transfected into a plant cell. Subsequently, a plant cell comprising the isolated nucleic acid is selected for by means known to those of skill in the art such as, but not limited to, Southern blot, DNA sequencing, or PCR analysis using primers specific to the promoter and to the nucleic acid and detecting amplicons produced therefrom. A plant or plant part altered or modified by the foregoing embodiments is grown under plant forming conditions for a time sufficient to modulate the concentration and/or composition of polypeptides of the present invention in the plant. Plant forming conditions are well known in the art.

In general, concentration of the polypeptides is increased or decreased by at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% relative to a native control plant, plant part, or cell lacking the aforementioned expression cassette. Modulation in the present invention may occur during and/or subsequent to growth of the plant to the desired stage of development.

Modulating nucleic acid expression temporally and/or in particular tissues can be controlled by employing the appropriate promoter operably linked to a polynucleotide of the present invention in, for example, sense or antisense orientation as discussed in greater detail above. Induction of expression of a polynucleotide of the present invention can also be controlled by exogenous administration of an effective amount of inducing compound. Inducible promoters and inducing compounds that activate expression from these promoters are well known in the art.

In preferred embodiments, the polypeptides of the present invention are modulated in monocots or dicots, preferably corn, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley, millet, *Arabidopsis thaliana*, tomato, *Brassica* vegetables, peppers, potatoes, apples, spinach, or lettuce.

Means of detecting the proteins of the present invention are not critical aspects of the present invention. In a preferred embodiment, the proteins are detected and/or quantified using any of a number of well recognized

immunological binding assays (see, e.g., U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168). For a review of the general immunoassays, see also *Methods in Cell Biology*, Vol. 37: *Antibodies in Cell Biology*, Asai, Ed., Academic Press, Inc. New York (1993); *Basic and Clinical Immunology* 7th Edition, Stites & Terr, Eds. (1991). Moreover, the immunoassays of the present invention can be performed in any of several configurations, e.g., those reviewed in *Enzyme Immunoassay*, Maggio, Ed., CRC Press, Boca Raton, Florida (1980); Tijan, Practice and Theory of Enzyme Immunoassays, *Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers B.V., Amsterdam (1985); Harlow and Lane, *supra*; *Immunoassay: A Practical Guide*, Chan, Ed., Academic Press, Orlando, FL (1987); *Principles and Practice of Immunoassays*, Price and Newman Eds., Stockton Press, NY (1991); and *Non-isotopic Immunoassays*, Ngo, Ed., Plenum Press, NY (1988).

Typical methods for detecting proteins include Western blot (immunoblot) analysis, analytic biochemical methods such as electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, and the like, and various immunological methods such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, and the like.

Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (e.g., biotin) is covalently bound to the molecule. The ligand then binds to an anti-ligand (e.g., streptavidin) molecule that is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. A number of ligands and anti-ligands can be used. Where a ligand has a natural anti-ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labeled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

The molecules can also be conjugated directly to signal generating compounds, e.g., by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives,

dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, e.g., luminol. For a review of various labeling or signal producing systems which may be used, see, U.S. Patent No. 4,391,904, which is incorporated herein by reference.

Some assay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of the target antibodies. In this case, antigen-coated particles are agglutinated by samples comprising the target antibodies. In this format, none of the components need be labeled and the presence of the target antibody is detected by simple visual inspection.

The proteins of the present invention can be used for identifying compounds that bind to (e.g., substrates), and/or increase or decrease (i.e., modulate) the enzymatic activity of, catalytically active polypeptides of the present invention. The method comprises contacting a polypeptide of the present invention with a compound whose ability to bind to or modulate enzyme activity is to be determined. The polypeptide employed will have at least 20%, preferably at least 30% or 40%, more preferably at least 50% or 60%, and most preferably at least 70% or 80% of the specific activity of the native, full-length polypeptide of the present invention (e.g., enzyme). Methods of measuring enzyme kinetics are well known in the art. See, e.g., Segel, *Biochemical Calculations*, 2<sup>nd</sup> ed., John Wiley and Sons, New York (1976).

Antibodies can be raised to a protein of the present invention, including individual, allelic, strain, or species variants, and fragments thereof, both in their naturally occurring (full-length) forms and in recombinant forms. Additionally, antibodies are raised to these proteins in either their native configurations or in non-native configurations. Anti-idiotypic antibodies can also be generated. Many methods of making antibodies are known to persons of skill.

In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans, etc. Description of techniques for preparing such monoclonal antibodies are found in, e.g., *Basic and Clinical Immunology*, 4th ed., Stites *et al.*, Eds., Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane, *Supra*; Goding, *Monoclonal Antibodies: Principles and Practice*, 2nd ed.,

Academic Press, New York, NY (1986); and Kohler and Milstein, *Nature* 256: 495-497 (1975).

Other suitable techniques involve selection of libraries of recombinant antibodies in phage or similar vectors (see, e.g., Huse *et al.*, *Science* 246:1275-1281 (1989); and Ward, *et al.*, *Nature* 341:544-546 (1989); and Vaughan *et al.*, *Nature Biotechnology*, 14:309-314 (1996)). Alternatively, high avidity human monoclonal antibodies can be obtained from transgenic mice comprising fragments of the unrearranged human heavy and light chain Ig loci (i.e., minilocus transgenic mice). Fishwild *et al.*, *Nature Biotech.*, 14:845-851 (1996). Also, recombinant immunoglobulins may be produced. See, Cabilly, U.S. Patent No. 4,816,567; and Queen *et al.*, *Proc. Nat'l Acad. Sci.* 86:10029-10033 (1989).

The antibodies of this invention can be used for affinity chromatography in isolating proteins of the present invention, for screening expression libraries for particular expression products such as normal or abnormal protein or for raising anti-idiotypic antibodies which are useful for detecting or diagnosing various pathological conditions related to the presence of the respective antigens.

Frequently, the proteins and antibodies of the present invention will be labeled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionucleotides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like.

### **Transformation of Cells**

The method of transformation/transfection is not critical to the invention; various methods of transformation or transfection are currently available. As newer methods are available to transform crops or other host cells they may be directly applied. Accordingly, a wide variety of methods have been developed to insert a DNA sequence into the genome of a host cell to obtain the transcription and/or translation of the sequence to effect phenotypic changes in the organism. Thus, any method that provides for efficient transformation/transfection may be employed.

A DNA sequence coding for the desired polynucleotide of the present invention, for example a cDNA, RNA or a genomic sequence, will be used to construct an expression cassette that can be introduced into the desired plant. Isolated nucleic acid acids of the present invention can be introduced into plants according techniques known in the art. Generally, expression cassettes as described above and suitable for transformation of plant cells are prepared.

Techniques for transforming a wide variety of higher plant species are well known and described in the technical, scientific, and patent literature. See, for example, Weising *et al.*, *Ann. Rev. Genet.* 22:421-477 (1988). For example, the DNA construct may be introduced directly into the genomic DNA of the plant cell using techniques such as electroporation, PEG-mediated transfection, particle bombardment, silicon fiber delivery, or microinjection of plant cell protoplasts or embryogenic callus. See, e.g., Tomes, *et al.*, Direct DNA Transfer into Intact Plant Cells Via Microprojectile Bombardment. pp.197-213 in Plant Cell, Tissue and Organ Culture, Fundamental Methods. eds. O. L. Gamborg and G.C. Phillips. Springer-Verlag Berlin Heidelberg New York, 1995. Alternatively, the DNA constructs may be combined with suitable T-DNA flanking regions and introduced into a conventional *Agrobacterium tumefaciens* host vector. The virulence functions of the *Agrobacterium tumefaciens* host will direct the insertion of the construct and adjacent marker into the plant cell DNA when the cell is infected by the bacteria. See, U.S. Patent No. 5,591,616.

The introduction of DNA constructs using polyethylene glycol precipitation is described in Paszkowski *et al.*, *Embo J.* 3:2717-2722 (1984). Electroporation techniques are described in Fromm *et al.*, *Proc. Natl. Acad. Sci.* 82:5824 (1985). Ballistic transformation techniques are described in Klein *et al.*, *Nature* 327:70-73 (1987).

*Agrobacterium tumefaciens*-mediated transformation techniques are well described in the scientific literature. See, for example Horsch *et al.*, *Science* 233:496-498 (1984), and Fraley *et al.*, *Proc. Natl. Acad. Sci.* 80:4803 (1983). For instance, *Agrobacterium* transformation of maize is described in U.S. Patent No. 5,550,318.

Other methods of transfection or transformation include (1) *Agrobacterium rhizogenes*-mediated transformation (see, e.g., Lichtenstein and Fuller In: Genetic Engineering, vol. 6, PWJ Rigby, Ed., London, Academic Press, 1987; and

Lichtenstein, C. P., and Draper, J., In: DNA Cloning, Vol. II, D. M. Glover, Ed., Oxford, IRI Press, 1985), Application PCT/US87/02512 (WO 88/02405 published Apr. 7, 1988) describes the use of *A. rhizogenes* strain A4 and its Ri plasmid along with *A. tumefaciens* vectors pARC8 or pARC16 (2) liposome-mediated DNA uptake (see, e.g., Freeman *et al.*, *Plant Cell Physiol.* 25:1353, 1984), (3) the vortexing method (see, e.g., Kindle, *Proc. Natl. Acad. Sci. USA* 87:1228, (1990).

DNA can also be introduced into plants by direct DNA transfer into pollen as described by Zhou *et al.*, *Methods in Enzymology*, 101:433 (1983); D. Hess, *Intern Rev. Cytol.*, 107:367 (1987); Luo *et al.*, *Plant Mol. Biol. Reporter*, 6:165 (1988). Expression of polypeptide coding nucleic acids can be obtained by injection of the DNA into reproductive organs of a plant as described by Pena *et al.*, *Nature* 325:274 (1987). DNA can also be injected directly into the cells of immature embryos and the rehydration of desiccated embryos as described by Neuhaus *et al.*, *Theor. Appl. Genet.*, 75:30 (1987); and Benbrook *et al.*, in *Proceedings Bio Expo 1986*, Butterworth, Stoneham, Mass., pp. 27-54 (1986).

Animal and lower eukaryotic (e.g., yeast) host cells are competent or rendered competent for transfection by various means. There are several well-known methods of introducing DNA into animal cells. These include: calcium phosphate precipitation, fusion of the recipient cells with bacterial protoplasts containing the DNA, treatment of the recipient cells with liposomes containing the DNA, DEAE dextran, electroporation, biolistics, and micro-injection of the DNA directly into the cells. The transfected cells are cultured by means well known in the art. Kuchler, R.J., *Biochemical Methods in Cell Culture and Virology*, Dowden, Hutchinson and Ross, Inc. (1977).

### **Transgenic Plant Regeneration**

Transformed plant cells which are derived by any of the above transformation techniques can be cultured to regenerate a whole plant which possesses the transformed genotype. Such regeneration techniques often rely on manipulation of certain phytohormones in a tissue culture growth medium, typically relying on a biocide and/or herbicide marker which has been introduced together with a polynucleotide of the present invention. For transformation and regeneration of maize see, Gordon-Kamm *et al.*, *The Plant Cell*, 2:603-618 (1990).



Plants cells transformed with a plant expression vector can be regenerated, e.g., from single cells, callus tissue or leaf discs according to standard plant tissue culture techniques. It is well known in the art that various cells, tissues, and organs from almost any plant can be successfully cultured to regenerate an entire plant. Plant regeneration from cultured protoplasts is described in Evans *et al.*, *Protoplasts Isolation and Culture, Handbook of Plant Cell Culture*, Macmillan Publishing Company, New York, pp. 124-176 (1983); and Binding, *Regeneration of Plants, Plant Protoplasts*, CRC Press, Boca Raton, pp. 21-73 (1985).

The regeneration of plants containing the foreign gene introduced by *Agrobacterium* can be achieved as described by Horsch *et al.*, *Science*, 227:1229-1231 (1985) and Fraley *et al.*, *Proc. Natl. Acad. Sci. USA* 80:4803 (1983). This procedure typically produces shoots within two to four weeks and these transformant shoots are then transferred to an appropriate root-inducing medium containing the selective agent and an antibiotic to prevent bacterial growth. Transgenic plants of the present invention may be fertile or sterile.

Regeneration can also be obtained from plant callus, explants, organs, or parts thereof. Such regeneration techniques are described generally in Klee *et al.*, *Ann. Rev. of Plant Phys.* 38:467-486 (1987). The regeneration of plants from either single plant protoplasts or various explants is well known in the art. See, for example, *Methods for Plant Molecular Biology*, A. Weissbach and H. Weissbach, eds., Academic Press, Inc., San Diego, Calif. (1988). For maize cell culture and regeneration see generally, *The Maize Handbook*, Freeling and Walbot, Eds., Springer, New York (1994); *Corn and Corn Improvement*, 3<sup>rd</sup> edition, Sprague and Dudley Eds., American Society of Agronomy, Madison, Wisconsin (1988).

One of skill will recognize that after the expression cassette is stably incorporated in transgenic plants and confirmed to be operable, it can be introduced into other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

In vegetatively propagated crops, mature transgenic plants can be propagated by the taking of cuttings or by tissue culture techniques to produce multiple identical plants. Selection of desirable transgenics is made and new varieties are obtained and propagated vegetatively for commercial use. In seed propagated crops, mature transgenic plants can be self crossed to produce a homozygous inbred plant. The inbred plant produces seed containing the newly

introduced heterologous nucleic acid. These seeds can be grown to produce plants that would produce the selected phenotype.

Parts obtained from the regenerated plant, such as flowers, seeds, leaves, branches, fruit, and the like are included in the invention, provided that these parts comprise cells comprising the isolated nucleic acid of the present invention. Progeny and variants, and mutants of the regenerated plants are also included within the scope of the invention, provided that these parts comprise the introduced nucleic acid sequences.

Transgenic plants expressing a selectable marker can be screened for transmission of the nucleic acid of the present invention by, for example, standard immunoblot and DNA detection techniques. Transgenic lines are also typically evaluated on levels of expression of the heterologous nucleic acid. Expression at the RNA level can be determined initially to identify and quantitate expression-positive plants. Standard techniques for RNA analysis can be employed and include PCR amplification assays using oligonucleotide primers designed to amplify only the heterologous RNA templates and solution hybridization assays using heterologous nucleic acid-specific probes. The RNA-positive plants can then be analyzed for protein expression by Western immunoblot analysis using the specifically reactive antibodies of the present invention. In addition, *in situ* hybridization and immunocytochemistry according to standard protocols can be done using heterologous nucleic acid specific polynucleotide probes and antibodies, respectively, to localize sites of expression within transgenic tissue. Generally, a number of transgenic lines are usually screened for the incorporated nucleic acid to identify and select plants with the most appropriate expression profiles.

A preferred embodiment is a transgenic plant that is homozygous for the added heterologous nucleic acid; i.e., a transgenic plant that contains two added nucleic acid sequences, one gene at the same locus on each chromosome of a chromosome pair. A homozygous transgenic plant can be obtained by sexually mating (selfing) a heterozygous transgenic plant that contains a single added heterologous nucleic acid, germinating some of the seed produced and analyzing the resulting plants produced for altered expression of a polynucleotide of the present invention relative to a control plant (i.e., native, non-transgenic). Back-

crossing to a parental plant and out-crossing with a non- transgenic plant are also contemplated.

The present invention provides a method of genotyping a plant comprising a polynucleotide of the present invention. Genotyping provides a means of distinguishing homologs of a chromosome pair and can be used to differentiate segregants in a plant population. Molecular marker methods can be used for phylogenetic studies, characterizing genetic relationships among crop varieties, identifying crosses or somatic hybrids, localizing chromosomal segments affecting monogenic traits, map based cloning, and the study of quantitative inheritance. See, e.g., *Plant Molecular Biology: A Laboratory Manual*, Chapter 7, Clark, Ed., Springer-Verlag, Berlin (1997). For molecular marker methods, see generally, *The DNA Revolution* by Andrew H. Paterson 1996 (Chapter 2) in: *Genome Mapping in Plants* (ed. Andrew H. Paterson) by Academic Press/R. G. Landis Company, Austin, Texas, pp.7-21.

The particular method of genotyping in the present invention may employ any number of molecular marker analytic techniques such as, but not limited to, restriction fragment length polymorphisms (RFLPs). RFLPs are the product of allelic differences between DNA restriction fragments caused by nucleotide sequence variability. Thus, the present invention further provides a means to follow segregation of a gene or nucleic acid of the present invention as well as chromosomal sequences genetically linked to these genes or nucleic acids using such techniques as RFLP analysis.

Plants that can be used in the method of the invention include monocotyledonous and dicotyledonous plants. Preferred plants include corn, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley, millet, *Arabidopsis thaliana*, tomato, *Brassica* vegetables, peppers, potatoes, apples, spinach, or lettuce.

Seeds derived from plants regenerated from transformed plant cells, plant parts or plant tissues, or progeny derived from the regenerated transformed plants, may be used directly as feed or food, or further processing may occur.

The present invention will be further described by reference to the following detailed examples. It is understood, however, that there are many extensions, variations, and modifications on the basic theme of the present invention beyond that shown in the examples and description, which are within the spirit and scope

of the present invention. All publications, patents, and patent applications cited herein are hereby incorporated by reference.

## EXAMPLES

### **Identification of a phytyl/prenyltransferase involved in biosynthesis of tocopherols in *Synechocystis* PCC 6803 and *Arabidopsis thaliana*.**

PCC 6803 was used as a tool for identification of genes encoding enzymes involved in biosynthesis of tocopherols. *Synechocystis* is a cyanobacterium capable of tocopherol biosynthesis. The entire genome of this photosynthetic organism has been recently sequenced (Kaneko et al., 1996) and the data is available on a public searchable database, called CyanoBase (<http://www.kazusa.or.jp/cyano/cyano.html>). Using CyanoBase, we have identified an open reading frame (SLR1736) encoding a phytyl/prenyltransferase involved in the biosynthesis of 2-methyl-6-phytylplastoquinol, one of the tocopherol precursors. This open reading frame was identified based on similarity with the phytyl/prenyltransferase SLR0056, a phytyl/prenyltransferase involved in the biosynthesis of chlorophyll in *Synechocystis* PCC 6803. SLR0056 exhibits a high homology with the previously identified chlorophyllide/phytyl/prenyltransferases from many cyanobacteria and *A. thaliana* (Lopez et al., 1996), suggesting that this enzyme is also involved in chlorophyll synthesis.

SLR1736 is similar, but not highly homologous to the SLR0056 open reading frame. However, the putative prenyl-binding domain is highly conserved in SLR1736, making it a good candidate for the tocopherol phytyl/prenyltransferase. Using the SLR1736 translated sequence as a query in the blast search, a genomic clone on chromosome II was identified in the *A. thaliana* database (Stanford Genomic Resources). This genomic clone was used to isolate an *Arabidopsis* cDNA clone. The F19F24 genomic clone and *Arabidopsis* cDNA are highly homologous to the SLR1736 protein sequence. The prenyl-binding domain is also conserved in the F19F24 and *Arabidopsis* cDNA. In addition, the amino terminal deduced amino acid sequence of the *Arabidopsis* gene and cDNA exhibits the traits of chloroplast-targeting sequences. Tocopherol biosynthesis has been shown to take place in chloroplast envelopes (Soll et al., 1980; Soll, 1987). We believe that the *Arabidopsis* F19F24 gene and homologous

cDNA represent the orthologous phytyl/prenyltransferase that attaches phytyldiphosphate (phytyl-PP) and/or geranylgeranyl pyrophosphate (GGPP) to homogentisic acid in tocopherol synthesis in *A. thaliana*. Additionally, a 1.2 kb corn EST, chste82, that is highly homologous to SLR1736 has been also identified in a blast search.

To demonstrate that SLR1736 might be involved in tocopherol biosynthesis in *Synechocystis*, this gene was disrupted by insertion of the kanamycin expression cassette. The method of gene disruption by gene replacement technique has been previously described (Williams, 1988). The resulting mutant was named  $\Delta$ SLR1736. Before analyses, the mutant was sub-cultured at least 6 times by single colony section on kanamycin to select for the colonies containing only copies of the SLR1736 gene disrupted with the kanamycin resistance gene. The absence of wild type SLR1736 genes was confirmed by PCR. The lack of tocopherols in the mutant was shown by HPLC separation of lipid extracts from wild type and mutant *Synechocystis* on a normal-phase column using fluorescent detection (FLD). Levels of phyloquinone (vitamin K1) and plastoquinone seem to be unaffected in this mutant. This suggests that there are at least two separate prenyltransferase activities for tocopherol and plastoquinone synthesis in *Synechocystis* and we may be able to manipulate carbon flow through the pathway by altering gene expression of either. Phytylation/prenylation of homogentisic acid is the branch-point in tocopherol and plastoquinone synthesis, and therefore, most likely an important regulatory point of the pathway. As well as the prenylation activities, availability of different prenyl tails may also be crucial for the regulation of carbon flow through the pathway. This will become significant for manipulating tocopherol levels in higher plants.

#### **Amplification of the SLR1736 open reading frame from *Synechocystis***

Chromosomal DNA from wild type *Synechocystis* PCC 6803 was isolated according to Williams (Methods in Enzymology (1987) 167: 766-778). The following primers were designed using Mac Vector computer program to amplify a 1.022 kb fragment containing the SLR1736 open reading frame:

SLR1736F: 5'-TATTCATATGGCAACTATCCAAGCTTTTGTG-3'

SLR1736R: 5'-GGATCCTAATTGAAGAAGATACTAAATAGTTC-3'

NdeI and BamHI sites were added to the primers to facilitate sub-cloning for expression purposes. ATG in the SLR1736F primer is the start codon for the SLR1736 open reading frame published in the CyanoBase Web-site. Taq polymerase (Gibco BRL) was used for gene disruption purposes and later Vent polymerase (NEB) was used for expression purposes following the manufacturer's recommendations. The following cycles were performed:

For Taq polymerase amplification:

95 °C/5 minutes (1 cycle)

95 °C/45 seconds, 45 °C/45 seconds, 68 °C/45 seconds (5 cycles)

95 °C/45 seconds, 52 °C/45 seconds, 72 °C/45 seconds (30 cycles)

72 °C/10 minutes

The same thermocycler conditions were used to amplify SLR1736 with Vent polymerase except that elongation times were extended to 2 minutes.

### **Sub-cloning the SRL1736 PCR product**

Plasmid pBluescript KS II (Stratagene) was digested with EcoRV (NEB) according to manufacturer's protocols. Both linearized pBluescript and the amplified SLR1736 open reading frame were separated by 0.9 % agarose TBE gel electrophoresis. The bands were excised and purified from the gel using a JetSorb DNA purification kit (PGC Scientifics). The purified fragment was sub-cloned into the EcoRV site of pBluescript KS II in a blunt-end ligation reaction. A 10 µl ligation reaction contained 20 mM Tris-HCl (pH 7.6), 5 mM MgCl<sub>2</sub>, 5 mM DTT, 50 µg/ml BSA, 0.5 mM rATP, 15 % PEG, and 1 U of T4 DNA ligase (Gibco BRL). Ligation was carried out at room temperature for 4 hours. One half of the reaction mixture was used to transform competent *E. coli* DH5α cells. Transformants were then selected on LB plates containing 100 mg/L of ampicillin. X-gal and IPTG were used for blue/white selection. White ampicillin resistant colonies were then selected, grown in liquid LB/ampicillin media, and plasmids were purified. The resulting plasmid was designated as KS-1736 and the nature and the orientation of the 1736 insert was determined by restriction digestion and sequencing (ABI Prism 310 Genetic Analyser). Clone #5, in which SLR1736 was in a reverse orientation to the Lac promoter of the vector, was selected for further manipulations.

### The SLR1736 replacement construct

Transformation followed by homologous recombination is feasible in *Synechocystis* (Williams, 1988). A gene of interest, in our case SLR1736, can be easily disrupted by inserting an antibiotic resistance gene into the coding region. Such a disruption construct can be transformed into *Synechocystis*. pBluescript KS or any other vector capable of replication in *E. coli* can be used as a vector. These vectors cannot be replicated by DNA replication machinery of *Synechocystis* so that the cells are forced to keep the resistance gene by other means when kept on the antibiotic selection. In *Synechocystis*, the wild type copies of the target gene are replaced with the copies of this gene disrupted with the antibiotic resistance cassette by homologous recombination. Since this cyanobacterium contains multiple copies of its genome, it is necessary to streak selected resistant colonies on the selection media several times. This should ensure replacement of the wild type copies of the gene with the disrupted ones (Williams, 1988).

The kanamycin resistance gene from the transposon Tn903 encoding aminoglycoside 3'-phosphotransferase was used to disrupt the wild type SLR1736 gene. Plasmid pUC4K (Pharmacia) was cut with EcoRI to release the kanamycin resistance expression cassette. Since SLR1736 has a unique MfeI site about 200 bp from the beginning of the gene, plasmid KS-1736 #5 was digested with MfeI (NEB). MfeI leaves 5'-cohesive ends compatible with EcoRI so that no other molecular manipulations are necessary. The two DNA fragments were purified from agarose gels as described above and ligated using T4 DNA ligase (Gibco BRL) as recommended by the manufacturer. Competent *E. coli* DH5 $\alpha$  cells were transformed with the ligation reaction and transformants selected on LB plates containing 50 mg of kanamycin per liter of media. Plasmids were purified and subjected to restriction analysis. Two plasmids having opposite orientation of the kanamycin cassette were chosen for *Synechocystis* transformation. The two constructs were designated as KS $\Delta$ 1736-KAN-F and B, respectively, to indicate the orientation of the kanamycin resistance gene in respect to the SLR1736 gene.

Transformation of *Synechocystis* PCC 6803 with KS $\Delta$ 1736-KAN-F and B, respectively, was carried out as described by Williams (1988). Transformants were selected on BG-11 plates containing 15 mM glucose and 5 mg of kanamycin

per liter of medium. Two independent colonies from each transformation were then sub-cultured once a week for several weeks on BG-11 plates containing 15 mM glucose and 15 mg/L kanamycin before being analyzed. The cells were grown under continuous light at 30°C. The resulting clones used for further analyses were designated  $\Delta$ SLR1736 F-1, F-2, B-1, and B-2.

### **Confirmation of the SLR1736 gene disruption by PCR**

Chromosomal DNA from wild type and  $\Delta$ SLR1736 mutant *Synechocystis* PCC 6803 was isolated from a few colonies according to Cai and Wolk (1990) with minor modifications as follows: Cells were resuspended in 200  $\mu$ l of 50 mM Tris.HCl and 10 mM EDTA solution of pH 7.5. The cells were then transferred to a 2 ml screw-cup tube. 10  $\mu$ l of 20% SDS, 200  $\mu$ l of phenol:chloroform (1:1), and white sand were added. The samples were mixed well by vortexing for 1 minute and then they were placed on ice for another minute. This step was repeated twice. The mixture was centrifuged at 14,000 rpm for 5 minutes to separate organic and aqueous layers. The top aqueous phase was then extracted twice with an equal volume of chloroform and precipitated with a quarter of volume of 3M potassium acetate (pH 4.8) and two volumes of 96% of ethanol. After an hour incubation at -20°C and a ten-minute centrifugation at 14,000 rpm, genomic DNA was washed once with 80% of ethanol, dried in a speedvac for 2-3 minutes, and resuspended in 20  $\mu$ l of water. DNA was diluted 1:10 with water and used as a template in PCR reactions. PCR was performed as described above using Taq polymerase (Gibco BRL). Insertion of the kanamycin cassette into the SLR1736 open reading frame was clearly demonstrated.

### **HPLC analyses of the lipid extracts from wild type and mutant *Synechocystis*.**

#### *Tocopherol analysis*

About 30 mg of wild type and  $\Delta$ SLR1736 F and B mutant cells grown on solid plates as described above were harvested and resuspended in 450  $\mu$ l of methanol: chloroform (2:1) containing 1 mg/ml of butylated hydroxytoluene (BHT) to prevent oxidation of tocopherols. 200 ng of tocol was added as an internal standard. The cells were homogenized using mini-pestals followed by addition of 150  $\mu$ l of chloroform and 300  $\mu$ l of water to the mixture. After centrifugation (5



minutes at 14,000 rpm), the lower organic phase was transferred to a clean microfuge tube and dried in a speedvac. Lipids were resuspended in 80  $\mu$ l of hexane containing 1 mg/ml of BHT. 40  $\mu$ l of the lipid extract was subjected to HPLC (Hewlett-Packard 1100 Series HPLC system with a fluorescence detector) using a normal phase column (Lichrosorb Si60A 4.6 X 250 mm) equilibrated at 42°C. A 20-minute linear gradient of 8 % to 18 % di-isopropyl ether in hexane was used to separate different types of tocopherols. After excitation at a wavelength of 290 nm, tocopherols were detected by their fluorescence at 325 nm.

Wild type *Synechocystis* accumulates predominantly  $\alpha$ -tocopherol. The  $\Delta$ SLR1736 disruption mutants lack all tocopherols and this effect is independent of the kanamycin cassette orientation. These results indicate that SLR1736 is involved in tocopherol biosynthesis and acts upstream of the methyltransferases. Disruption of the methyltransferase genes SLL0418 (2-methyl-6-phytylplastoquinol methyltransferase) and SLR0089 ( $\gamma$ -tocopherol methyltransferase) which have been recently cloned from *Synechocystis* leads to the accumulation of  $\beta$ - and  $\gamma$ -tocopherols, respectively (Shintani, D., personal communication; Shintani and DellaPenna, 1998). The only two possible remaining enzymes are the cyclase and prenyltransferase. Since SLR1736 exhibits a similarity to known prenyltransferases, we believe this enzyme represents a prenyltransferase. More conclusive proof than the one based on similarity would be given by in vitro prenyltransferase assays and feeding studies of wild type and  $\Delta$ SLR1736 mutant *Synechocystis* with  $^{14}\text{C}$  uniformly labeled tyrosine.

#### *Phylloquinone and plastoquinone analysis*

Formation of homogentisic acid, the first step of the pathway, is common for both tocopherols and plastoquinone in photosynthetic organisms. To answer the question if the tocopherol prenyltransferase is also involved in plastoquinone biosynthesis and how carbon flow is affected in the plastoquinone part of the pathway, we analyzed lipid extracts from wild type and the mutant cells. On the other hand, the phytyl tail is a part of vitamin K1 (phylloquinone) molecule. To estimate effects of the SLR1736 gene disruption on the phylloquinone biosynthesis in *Synechocystis*, we also performed vitamin K1 analysis.

About 30 mg of wild type and  $\Delta$ SLR1736 F and B mutant were harvested and resuspended in 450  $\mu$ l of methanol: chloroform (2:1). The cells were homogenized using mini-pestals followed by addition of 150  $\mu$ l of chloroform and 300  $\mu$ l of water to the mixture. After centrifugation (5 minutes at 14,000 rpm), the lower organic phase was transferred to a clean microfuge tube, dried in a speedvac, dissolved in 30  $\mu$ l of ethyl acetate, and oxidized with silver oxide for a half an hour. The entire extract was loaded on a TLC plate (Silica, 60A) which was developed in 20% diethyl ether in petroleum ether and dried. The plate was sprayed with leucomethylene blue (Crane & Barr, 1971) to visualize any changes in quinone composition. No differences between the wild type and mutant quinone profiles were observed. To prepare leucomethylene blue, 50 mg of methylene blue and 0.5 g of zinc dust were mixed in 5 ml of water. The mixture was acidified with a few drops of concentrated sulfuric acid and left to react for about 10 minutes before use.

To quantify possible changes in quinone content in wild type and mutant *Synechocystis*, HPLC analyses of lipid extracts containing plastoquinone-8 as an internal standard were performed. Lipids were extracted as described above except that 1  $\mu$ g of plastoquinone-8 was added in the beginning of extraction. Plastoquinone-8 and plastoquinone-9 standards were isolated and purified from *Iris holandica* bulbs (Hutson & Therfall, 1980) and their concentrations were determined using the molar absorption coefficient of plastoquinone-9 at 254 nm,  $17.94 \text{ mM}^{-1} \text{ cm}^{-1}$ . These quinones have similar properties and they can be easily separated by the HPLC method described below. Therefore, plastoquinone-8 is an excellent internal standard.

After extraction, quinones were resuspended in 80  $\mu$ l of HPLC grade ethyl acetate. 40  $\mu$ l of the lipid extract was subjected to HPLC (Hewlett-Packard 1100 Series HPLC system) using a C-18 reverse phase column (Spherisorb, 4.6 X 250 mm). The following conditions were utilized to separate different quinones:

Time (min.):	Reagent Alcohol (%):	Water (%):
0	90	10
1	99	1
10	99	1
11	100	0
16	100	0
17	90	10
35	90	10

The flow of the solvents was 0.8 ml/min and the separation was performed at room temperature. Quinones were detected by their absorbance at 250 and 275 nm using a diode array detector and the identity of phyloquinone and plastoquinone-9 was confirmed by comparison with their previously published spectra (Crane & Barr, 1971). No differences in vitamin K1 and plastoquinone-9 compositions were observed between wild type and the  $\Delta$ SLR1736 disruption mutant. This indicates that the SLR1736 gene product is involved only in tocopherol biosynthesis.

### **Cloning phytyl transferase from *A. thaliana* involved in tocopherol biosynthesis**

A developing seed-specific cDNA library from *A. thaliana* (lambda-ZAP type, provided by John Ohlrogge at the Michigan State University) was screened using a PCR product from wild type *A. thaliana* genomic DNA (Ler ecotype) which exhibits a high degree of homology with the *Synechocystis* phytyl transferase. Primers AT1736F (5'-TTGTTTTCAGGCTGTTGTTGCAGCTCTC-3') and AT1736B (5'-CGTTTCTGACCCAGAGTTACAGAGAATG-3') were used to amplify about 1kb fragment corresponding to 60238 – 61229 bp region of the BAC clone F19F24 (*A. thaliana* database at Stanford). The following program was used to amplify this fragment with Vent DNA polymerase (New England Biolabs):

95 °C/5 minutes (1 cycle)

95 °C/45 seconds; 50 °C/45 seconds, 72 °C/1 minute (30 cycles)

72 °C/10 minutes (1 cycle)

The PCR product was then sub-cloned into *EcoRV* site of pBluescript KS (Stratagene) as in the case of the cyanobacterial phytyl transferase presented above and sequenced from both ends using T3 and T7 primers (Stratagene) to

ensure the identity of the sub-cloned fragment. A 300 bp fragment of the insert (5'-end) was released with *EcoRI* from the vector and used as a radioactively-labeled probe to obtain full-length clones. About 2.5 million plaques of the seed-specific library were screened using standard procedures (Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989). *Molecular Cloning*. 2<sup>nd</sup> edition, Cold Spring Harbor Laboratory Press). 16 positive non-purified plaques were chosen for PCR analysis using T3 and AT1736T7c (5'-GACATATTTTGCAGTCTGCC-3) which is an internal primer for the phytyl transferase. Clones #1, 3, 5, 8, 11, 12, and 14 were selected for further purification and single clone excision, performed according to manufacturer (Stratagene), to obtain individual clones in pBluescript SK plasmids. Each clone was sequenced from each end using T3 and T7 primer. The longest clone, #11 – about 1.6 kb, was chosen for complete sequencing which is in progress now. All clones were aligned to the genomic clone F19F24 from *A. thaliana* to confirm their identity, identify introns and find possible sequencing mistakes in the genomic sequence. We believe that ATG codon (59220 bp on F19F24) is the start codon of the phytyl transferase involved in tocopherol synthesis in *A. thaliana*. Starting from this methionine, the first 36 amino acids represent the chloroplast thylakoid membrane-targeting sequence (PSORT program, <http://psort.nibb.ac.jp:8800/>).

### **Confirmation of prenyltransferase nature of SLR1736**

To confirm the prenyltransferase nature of SLR1736, the intact gene will be expressed in *E. coli* because this bacterium lacks any enzymatic activity connected to tocopherol biosynthesis. Therefore, SLR1736 activity will be shown by an in vitro phytyl/prenyltransferase assay using protein extracts from *E. coli* expressing SLR1736 or by reconstruction of multiple steps of the pathway in *E. coli*. <sup>14</sup>C uniformly labeled p-hydroxyphenyl pyruvate and phytyl-PP, or other prenyl diphosphates will be used as substrates. p-hydroxyphenyl pyruvate dioxygenase catalyses conversion of p-hydroxyphenylpyruvic acid to homogentisic acid, the immediate substrate for the tocopherol and plastoquinone prenyltransferase(s). Therefore, *A. thaliana* p-hydroxyphenylpyruvic acid dioxygenase (Norris *et al.*, 1998) expressed in *E. coli* along with the prenyltransferase will be present in the reactions to couple the two enzymatic steps. To further show that SLR1736 is a prenyltransferase,  $\Delta$ SLR1736 and wild

type *Synechocystis* will be grown in the presence of  $^{14}\text{C}$  uniformly labeled L-tyrosine to trace prenylated products by using TLC and autoradiography.

The SLR1736 open reading frame will be also expressed in *E. coli* in the presence of p-hydroxyphenylpyruvic acid dioxygenase (Norris *et al.*, 1998), *Adonis paleastina* geranylgeranyl diphosphate synthase (gift from F. Cunningham), and geranylgeranyl hydrogenase from *Synechocystis* (SLL1091, Addlesee *et al.*, 1996; Keller *et al.*, 1998) to reconstitute the phytol pyrophosphate pathway since *E. coli* does not possess any of these enzymatic activities. Lipids will be extracted and subjected to HPLC analysis of quinones as described above. 2-methyl-6-phytylplastoquinone is stable and should be present in *E. coli* lipid extracts.

### **SLR1736 homologue from *A. thaliana* (AT1736)**

To investigate the role of the plant homologue of SLR1736, the intact full length cDNA from *Arabidopsis thaliana* (AT1736) and corn chste82 EST will be expressed in the sense and antisense orientation from the constitutive CaMV 35S or seed-specific (Seffens *et al.*, 1990) promoters, respectively, in *A. thaliana*. Visible phenotype(s) will be observed and lipids from the transgenic plants will be extracted and subjected to HPLC/FLD analyses to detect changes in tocopherol content and composition in green tissues and seeds. Plastoquinone and phyloquinone levels will also be analyzed as described above. It is possible that phytol-PP is limiting for the prenyltransferase activity. Consequently, we may want to overexpress geranylgeranyl pyrophosphate synthase and GGDP dehydrogenase to elevate phytol-PP levels in *A. thaliana*. Columbia ecotype *Arabidopsis* plants will be transformed with these overexpression constructs separately and homozygous transformants will be crossed to obtain plants containing all three constructs.

The *in vitro* prenyltransferase assay will be performed with AT1736 expressed in *E. coli* as described above for SLR1736. Prenyl tail-specificity studies will be also carried out with this enzyme, using various tails such as GGDP, phytol-PP, and solanyl-PP. As in the case of SLR1736 from *Synechocystis*, it is important to determine if there are one or two prenyltransferases for tocopherol and plastoquinone biosynthesis in higher plants.

## Construction of p0018 *Maize* cDNA libraries

### Total RNA Isolation

Total RNA was isolated from p0018 library corn tissues with TRIzol Reagent (Life Technology Inc. Gaithersburg, MD) using a modification of the guanidine isothiocyanate/acid-phenol procedure described by Chomczynski and Sacchi (Chomczynski, P., and Sacchi, N. Anal. Biochem. 162, 156 (1987)). In brief, plant tissue samples were pulverized in liquid nitrogen before the addition of the TRIzol Reagent, and then were further homogenized with a mortar and pestle. Addition of chloroform followed by centrifugation was conducted for separation of an aqueous phase and an organic phase. The total RNA was recovered by precipitation with isopropyl alcohol from the aqueous phase.

### Poly(A)+ RNA Isolation

The selection of poly(A)+ RNA from total RNA was performed using PolyATact system (Promega Corporation. Madison, WI). In brief, biotinylated oligo(dT) primers were used to hybridize to the 3' poly(A) tails on mRNA. The hybrids were captured using streptavidin coupled to paramagnetic particles and a magnetic separation stand. The mRNA was washed at high stringent condition and eluted by RNase-free deionized water.

### cDNA Library Construction

cDNA synthesis was performed and unidirectional cDNA libraries were constructed using the SuperScript Plasmid System (Life Technology Inc. Gaithersburg, MD). The first strand of cDNA was synthesized by priming an oligo(dT) primer containing a Not I site. The reaction was catalyzed by SuperScript Reverse Transcriptase II at 45°C. The second strand of cDNA was labeled with alpha-32P-dCTP and a portion of the reaction was analyzed by agarose gel electrophoresis to determine cDNA sizes. cDNA molecules smaller than 500 base pairs and unligated adapters were removed by Sephacryl-S400 chromatography. The selected cDNA molecules were ligated into pSPORT1 vector in between of Not I and Sal I sites.

## Sequencing of Maize cDNA and Library Subtraction

### Sequencing Template Preparation

Individual colonies were picked and DNA was prepared either by PCR with M13 forward primers and M13 reverse primers, or by plasmid isolation. All the cDNA clones were sequenced using M13 reverse primers.

### Q-bot Subtraction Procedure

cDNA libraries subjected to the subtraction procedure are plated out on 22 x 22 cm<sup>2</sup> agar plate at density of about 3,000 colonies per plate. The plates are incubated in a 37°C incubator for 12-24 hours. Colonies are picked into 384-well plates by a robot colony picker, Q-bot (GENETIX Limited). These plates are incubated overnight at 37°C.

Once sufficient colonies are picked, they were pinned onto 22 x 22 cm<sup>2</sup> nylon membranes using Q-bot. Each membrane contained 9,216 colonies or 36,864 colonies. These membranes are placed onto agar plate with appropriate antibiotic. The plates are incubated at 37°C for overnight.

After colonies are recovered on the second day, these filters are placed on filter paper prewetted with denaturing solution for four minutes, then are incubated on top of a boiling water bath for additional four minutes. The filters are then placed on filter paper prewetted with neutralizing solution for four minutes. After excess solution is removed by placing the filters on dry filter papers for one minute, the colony side of the filters are placed into Proteinase K solution, incubated at 37°C for 40-50 minutes. The filters are placed on dry filter papers to dry overnight. DNA is then cross-linked to nylon membrane by UV light treatment.

Colony hybridization is conducted as described by Sambrook, J., Fritsch, E.F. and Maniatis, T., (in *Molecular Cloning: A laboratory Manual*, 2nd Edition). The following probes were used in colony hybridization:

1. First strand cDNA from the same tissue as the library was made from to remove the most redundant clones.
2. 48-192 most redundant cDNA clones from the same library based on previous sequencing data.
3. 192 most redundant cDNA clones in the entire corn sequence database.
4. A Sal-A20 oligo nucleotide: TCG ACC CAC GCG TCC GAA AAA AAA AAA AAA AAA AAA, removes clones containing a poly A tail but no cDNA.

## 5. cDNA clones derived from rRNA.

The image of the autoradiography is scanned into computer and the signal intensity and cold colony addresses of each colony is analyzed. Re-arraying of cold-colonies from 384 well plates to 96 well plates is conducted using Q-bot.

### **Identification of Gene from a Computer Homology Search**

Gene identities were determined by conducting BLAST (Basic Local Alignment Search Tool; Altschul, S. F., *et al.*, (1993) J. Mol. Biol. 215:403-410; see also [www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)) searches under default parameters for similarity to sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein Data Bank, the last major release of the SWISS-PROT protein sequence database, EMBL, and DDBJ databases). The cDNA sequences were analyzed for similarity to all publicly available DNA sequences contained in the "nr" database using the BLASTN algorithm provided by the National Center for Biotechnology Information (NCBI). The DNA sequences were translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the "nr" database using the BLASTX algorithm (Gish, W. and States, D. J. Nature Genetics 3:266-272 (1993)) provided by the NCBI. In some cases, the sequencing data from two or more clones containing overlapping segments of DNA were used to construct contiguous DNA sequences.



## Construction of Additional *Maize, Rice, Soybean and Wheat* cDNA libraries

### Composition of cDNA Libraries; Isolation and Sequencing of cDNA Clones

cDNA libraries representing mRNAs from maize, rice, soybean and wheat tissues were prepared. The characteristics of these libraries are described in

Table 1.

TABLE 1		
Library Designation	Library Description	Clone
cco1n	Corn Cob of 67 Day Old Plants Grown in Green House*	cco1n.pk087.117
p0018	Seedling after 10 day drought (T001), heat shocked for 24 hrs (T002), recovery at normal growth condition for 8 hrs, 16 hrs, 24hrs	p0018.chste82r:fis
p0108	PR leaves + C.carbonium, screened 1 Pool of PR+C. carbonium tox-3h; PR+C. carbonium tox-6h; PR+C. carbonium tox-24h; PR+C. carbonium tox-48hr; and PR+C. carbonium tox-7 7 days	p0108.cjrnc89r:fis
rca1n	Rice ( <i>Oryza sativa</i> L., Nipponbare) callus normalized.	rca1n.pk025.c4
rl0n	Rice 15 Day Old Leaf*	rl0n.pk0066.e2:fis
scr1c	Soybean ( <i>Glycine max</i> L., 2872) Embryogenic suspension culture subjected to 4 vacuum cycles and collected 12 hrs later (control scb1c).	scr1c.pk005.12
sgc7c	Soybean ( <i>Glycine max</i> L., Wye) germanating cotyledon (yellow and wilting; 18-30 DAG).	sgc7c.pk001.h22
src2c	Soybean ( <i>Glycine max</i> L., 437654) 8 day old root inoculated with eggs of cyst Nematode (Race 1) for 4 days.	src2c.pk020.d5:fis
wdk2c	Wheat Developing Kernel, 7 Days After Anthesis.	wdk2c.pk012.f2
wlm0	Wheat Seedlings 0 Hour After Inoculation With <i>Erysiphe graminis f. sp tritici</i>	wlm0.pk0011.c7

\*These libraries were normalized essentially as described in U.S. Pat. No. 5,482,845, incorporated herein by reference.

In general, cDNA libraries may be prepared by the method described above or by any one of many other methods available. For example, the cDNAs may be introduced into plasmid vectors by first preparing the cDNA libraries in Uni-ZAP™ XR vectors according to the manufacturer's protocol (Stratagene Cloning Systems, La Jolla, CA). The Uni-ZAP™ XR libraries are converted into plasmid libraries according to the protocol provided by Stratagene. Upon conversion, cDNA inserts will be contained in the plasmid vector pBluescript. In addition, the cDNAs may be introduced directly into precut Bluescript II SK(+) vectors (Stratagene) using T4 DNA ligase (New England Biolabs), followed by transfection into DH10B cells according to the manufacturer's protocol (GIBCO BRL Products). Once the cDNA inserts are in plasmid vectors, plasmid DNAs are prepared from randomly picked bacterial colonies containing recombinant pBluescript plasmids, or the insert cDNA sequences are amplified via polymerase chain reaction using primers specific for vector sequences flanking the inserted cDNA sequences.

Amplified insert DNAs or plasmid DNAs are sequenced in dye-primer sequencing reactions to generate partial cDNA sequences (expressed sequence tags or “ESTs”; see Adams et al., (1991) *Science* 252:1651-1656). The resulting ESTs are analyzed using a Perkin Elmer Model 377 fluorescent sequencer.

#### Characterization of cDNA Clones Encoding Phytyl/prenyltransferase.

cDNA Clones were identified by computer homology search as described above. The BLASTP and BLASTN searches using the sequences from clones listed in Table 1 revealed similarity to certain polypeptides as shown in Table 2. The “/blast/data/2.0/2/nr” database was searched. GAP results showing % identity to synechocystis and arabidopsis are also shown. Table 2 shows the BLAST results for individual complete gene sequences (“CGS”).

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**TABLE 2**

**Top BLAST Results for Sequences Encoding Polypeptides Homologous to Phytyl/prenyltransferase and GAP % Identity to Synechocystis and Arabidopsis**

Clone	Status	Protein Sequence with Significant Alignment gi# (accession #) Organism; % Blast Identity	GAP % Identity  Clone to D90909	GAP % Identity  Clone to AC003673
SEQ ID 12 - Contig of: cco1n.pk087.117 and cen3n.pk0012.h6	CGS	1652856 (D90909) Synechocystis; 36% 3004556 (AC003673) Arabidopsis; 32%	36.80%	50.27%
SEQ ID 4 - p0018.chste82r.fis	CGS	1652856 (D90909) Synechocystis; 43% 3004556 (AC003673) Arabidopsis; 47%	43.58%	70.67%
SEQ ID 14 - p0108.cjrnc89r.fis	CGS	1652856 (D90909) Synechocystis; 36% 6015890 (Y18930) Sulfolobus; 30% 5103549 (AP000058) Aeropyrum; 32% 3004556 (AC003673) Arabidopsis; 26%	37.54%	30.30%
SEQ ID 16 - rcaln.pk025.c4	CGS	1652856 (D90909) Synechocystis; 45% 3004556 (AC003673) Arabidopsis; 43%	45.27%	70.67%
SEQ ID 18 - r10n.pk0066.e2.fis	CGS	1652856 (D90909) Synechocystis; 35% 5103549 (AP000058) Aeropyrum; 29% 6015890 (Y18930) Sulfolobus; 28% 3004556 (AC003673) Arabidopsis; 25%	35.59%	31.16%
SEQ ID 20 - scr1c.pk005.12	CGS	1652856 (D90909) Synechocystis; 37% 6015890 (Y18930) Sulfolobus; 28% 3004556 (AC003673) Arabidopsis; 25%	36.95%	33.33%
SEQ ID 22 - Contig of: sgc7c.pk001.h22	CGS	1652856 (D90909) Synechocystis; 44% 3004556 (AC003673) Arabidopsis; 83%	44.90%	75.48%
SEQ ID 24 - src2c.pk020.d5.fis	CGS	3004556 (AC003673) Arabidopsis; 39% 1652856 (D90909) Synechocystis; 29%	30.51%	52.62%
SEQ ID 26 - Contig of: wdk2c.pk012.f2	Partial Gene Seq	3004556 (AC003673) Arabidopsis; 45% 1652856 (D90909) Synechocystis; 37%	37.50%	43.75%
SEQ ID 28- Contig of: wlm0.pk0011.c7	CGS	1652856 (D90909) Synechocystis; 36% 3004556 (AC003673) Arabidopsis; 27%	37.54%	30.30%

Sequence alignments and BLAST sequence identities indicate that the nucleic acid fragments comprising the instant cDNA clones encode a substantial portion of a phytyl/prenyltransferase.

A BLASTN search of "/blast/data/2.0/3/est" using the sequences from clones listed in Table 1 showed homology (E score > 140) to the following sequences on Table 3 as indicated by Genbank Accession number.

TABLE 3 - GAP Search Result

Number	Species	Shows Homology With Seq ID No.
C25006	Rice	17
C74444	Rice	15
AA750728	Rice	15
AA749638	Rice	15
AU029707	Rice	15
AI612332	Corn	13
AI711952	Corn	13
AI795680	Corn	13
AI897027	Tomato	21
AI938270	Soybean	21
AI938569	Soybean	21
		23
AI948381	Corn	13
AW052841	Corn	13
AW054141	Corn	11
AW066179	Corn	11
AW146615	Corn	13,
		17
AW202246	Soybean	19
AI444024	Soybean	19
AI442111	Soybean	19
AW132909	Soybean	23
		21
AI748688	Soybean	21
AI939002	Soybean	19
AW306617	Soybean	21
AW433064	Soybean	21
AW563431	Sorghum	17

In sequencing clone containing SEQ ID NO: 11, an extra nucleotide at nt 631 was observed. In addition, possible frameshifts at nt 107-140 were located that may interrupt homology to the *Synechocystis* hypothetical protein # 1652856. The extra nucleotide at nt 631 was deleted from the sequence listing provided with this application, and sequence identity was determined without considering the extra nucleotide. The extra nucleotide is likely an artifact occurring during the isolation and sequencing of the cDNA clones.

Clones p0108.cjrmc89r:fis and r10n.pk0066.e2:fis each contain substantially complete gene sequence, with the exception of a few N-terminal amino acids on each.

Clone wdk2c.pk012.f2 has an apparent intron from nt 322 to 426, as determined by GT/AG intron borders, that interrupt homology to p0018.chste82r:fis. The sequence listing provides both the nucleotide sequence of the clone with the intron (SEQ ID NO: 29) and without the intron (SEQ ID NO: 25). Amino acid sequence identity in SEQ ID NO: 26 was determined after removal of the intron.

The amino acid sequence of clone wlm0.pk0011.c7 covers the entire phytyl/prenyltransferase and contains a putative transit peptide sequence.

#### Expression of Chimeric Genes in Monocot Cells

A chimeric gene comprising a cDNA encoding the instant polypeptides in sense orientation with respect to the maize 27 kD zein promoter that is located 5' to the cDNA fragment, and the 10 kD zein 3' end that is located 3' to the cDNA fragment, can be constructed. The cDNA fragment of this gene may be generated by polymerase chain reaction (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites (NcoI or SmaI) can be incorporated into the oligonucleotides to provide proper orientation of the DNA fragment when inserted into the digested vector pML103 as described below. Amplification is then performed in a standard PCR. The amplified DNA is then digested with restriction enzymes NcoI and SmaI and fractionated on an agarose gel. The appropriate band can be isolated from the gel and combined with a 4.9 kb NcoI-SmaI fragment of the plasmid pML103. Plasmid pML103 has been deposited under the terms of the Budapest Treaty at ATCC (American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209), and bears accession number ATCC 97366. The DNA segment from pML103 contains a 1.05 kb Sall-NcoI promoter fragment of the maize 27 kD zein gene and a 0.96 kb SmaI-Sall fragment from the 3' end of the maize 10 kD zein gene in the vector pGem9Zf(+) (Promega). Vector and insert DNA can be ligated at 15°C overnight, essentially as described (Maniatis). The ligated DNA may then be used to transform *E. coli*

XL1-Blue (Epicurian Coli XL-1 Blue™; Stratagene). Bacterial transformants can be screened by restriction enzyme digestion of plasmid DNA and limited nucleotide sequence analysis using the dideoxy chain termination method (Sequenase™ DNA Sequencing Kit; U.S. Biochemical). The resulting plasmid construct would comprise a chimeric gene encoding, in the 5' to 3' direction, the maize 27 kD zein promoter, a cDNA fragment encoding the instant polypeptides, and the 10 kD zein 3' region.

The chimeric gene described above can then be introduced into corn cells by the following procedure. Immature corn embryos can be dissected from developing caryopses derived from crosses of the inbred corn lines H99 and LH132. The embryos are isolated 10 to 11 days after pollination when they are 1.0 to 1.5 mm long. The embryos are then placed with the axis-side facing down and in contact with agarose-solidified N6 medium (Chu et al. (1975) *Sci. Sin. Peking* 18:659-668). The embryos are kept in the dark at 27°C. Friable embryogenic callus consisting of undifferentiated masses of cells with somatic proembryoids and embryoids borne on suspensor structures proliferates from the scutellum of these immature embryos. The embryogenic callus isolated from the primary explant can be cultured on N6 medium and sub-cultured on this medium every 2 to 3 weeks.

The plasmid, p35S/Ac (obtained from Dr. Peter Eckes, Hoechst Ag, Frankfurt, Germany) may be used in transformation experiments in order to provide for a selectable marker. This plasmid contains the *Pat* gene (see European Patent Publication 0 242 236) which encodes phosphinothricin acetyl transferase (PAT). The enzyme PAT confers resistance to herbicidal glutamine synthetase inhibitors such as phosphinothricin. The *pat* gene in p35S/Ac is under the control of the 35S promoter from Cauliflower Mosaic Virus (Odell et al. (1985) *Nature* 313:810-812) and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*.

The particle bombardment method (Klein et al. (1987) *Nature* 327:70-73) may be used to transfer genes to the callus culture cells. According to this method, gold particles (1 µm in diameter) are coated with DNA using the following technique. Ten µg of plasmid DNAs are added to 50 µL of a suspension of gold particles (60 mg per mL). Calcium chloride (50 µL of a 2.5 M solution) and spermidine free base (20 µL of a 1.0 M solution) are added to the particles. The

suspension is vortexed during the addition of these solutions. After 10 minutes, the tubes are briefly centrifuged (5 sec at 15,000 rpm) and the supernatant removed. The particles are resuspended in 200  $\mu$ L of absolute ethanol, centrifuged again and the supernatant removed. The ethanol rinse is performed again and the particles resuspended in a final volume of 30  $\mu$ L of ethanol. An aliquot (5  $\mu$ L) of the DNA-coated gold particles can be placed in the center of a Kapton™ flying disc (Bio-Rad Labs). The particles are then accelerated into the corn tissue with a Biolistic™ PDS-1000/He (Bio-Rad Instruments, Hercules CA), using a helium pressure of 1000 psi, a gap distance of 0.5 cm and a flying distance of 1.0 cm.

For bombardment, the embryogenic tissue is placed on filter paper over agarose-solidified N6 medium. The tissue is arranged as a thin lawn and covered a circular area of about 5 cm in diameter. The petri dish containing the tissue can be placed in the chamber of the PDS-1000/He approximately 8 cm from the stopping screen. The air in the chamber is then evacuated to a vacuum of 28 inches of Hg. The macrocarrier is accelerated with a helium shock wave using a rupture membrane that bursts when the He pressure in the shock tube reaches 1000 psi.

Seven days after bombardment the tissue can be transferred to N6 medium that contains gluphosinate (2 mg per liter) and lacks casein or proline. The tissue continues to grow slowly on this medium. After an additional 2 weeks the tissue can be transferred to fresh N6 medium containing gluphosinate. After 6 weeks, areas of about 1 cm in diameter of actively growing callus can be identified on some of the plates containing the glufosinate-supplemented medium. These calli may continue to grow when sub-cultured on the selective medium.

Plants can be regenerated from the transgenic callus by first transferring clusters of tissue to N6 medium supplemented with 0.2 mg per liter of 2,4-D. After two weeks the tissue can be transferred to regeneration medium (Fromm et al. (1990) *Bio/Technology* 8:833-839).

#### Expression of Chimeric Genes in Dicot Cells

A seed-specific expression cassette composed of the promoter and transcription terminator from the gene encoding the  $\beta$  subunit of the seed storage protein phaseolin from the bean *Phaseolus vulgaris* (Doyle et al. (1986) *J. Biol.*

*Chem.* 261:9228-9238) can be used for expression of the instant polypeptides in transformed soybean. The phaseolin cassette includes about 500 nucleotides upstream (5') from the translation initiation codon and about 1650 nucleotides downstream (3') from the translation stop codon of phaseolin. Between the 5' and 3' regions are the unique restriction endonuclease sites Nco I (which includes the ATG translation initiation codon), Sma I, Kpn I and Xba I. The entire cassette is flanked by Hind III sites.

The cDNA fragment of this gene may be generated by polymerase chain reaction (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites can be incorporated into the oligonucleotides to provide proper orientation of the DNA fragment when inserted into the expression vector. Amplification is then performed as described above, and the isolated fragment is inserted into a pUC18 vector carrying the seed expression cassette.

Soybean embryos may then be transformed with the expression vector comprising sequences encoding the instant polypeptides. To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface sterilized, immature seeds of the soybean cultivar A2872, can be cultured in the light or dark at 26°C on an appropriate agar medium for 6-10 weeks. Somatic embryos which produce secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos which multiplied as early, globular staged embryos, the suspensions are maintained as described below.

Soybean embryogenic suspension cultures can be maintained in 35 mL liquid media on a rotary shaker, 150 rpm, at 26°C with fluorescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 mL of liquid medium.

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein et al. (1987) *Nature* (London) 327:70-73, U.S. Patent No. 4,945,050). A DuPont Biolistic™ PDS1000/HE instrument (helium retrofit) can be used for these transformations.

A selectable marker gene which can be used to facilitate soybean transformation is a chimeric gene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell et al. (1985) *Nature* 313:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz et al. (1983) *Gene* 25:179-188) and the 3' region of the nopaline synthase gene from the



T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The seed expression cassette comprising the phaseolin 5' region, the fragment encoding the instant polypeptides and the phaseolin 3' region can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

To 50  $\mu\text{L}$  of a 60 mg/mL 1  $\mu\text{m}$  gold particle suspension is added (in order): 5  $\mu\text{L}$  DNA (1  $\mu\text{g}/\mu\text{L}$ ), 20  $\mu\text{L}$  spermidine (0.1 M), and 50  $\mu\text{L}$   $\text{CaCl}_2$  (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400  $\mu\text{L}$  70% ethanol and resuspended in 40  $\mu\text{L}$  of anhydrous ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five  $\mu\text{L}$  of the DNA-coated gold particles are then loaded on each macro carrier disk.

Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5-10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as described above.

Five to seven days post bombardment, the liquid media may be exchanged with fresh media, and eleven to twelve days post bombardment with fresh media containing 50 mg/mL hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post bombardment, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

### Expression of Chimeric Genes in Microbial Cells

The cDNAs encoding the instant polypeptides can be inserted into the T7 *E. coli* expression vector pBT430. This vector is a derivative of pET-3a (Rosenberg et al. (1987) *Gene* 56:125-135) which employs the bacteriophage T7 RNA polymerase/T7 promoter system. Plasmid pBT430 was constructed by first destroying the EcoR I and Hind III sites in pET-3a at their original positions. An oligonucleotide adaptor containing EcoR I and Hind III sites was inserted at the BamH I site of pET-3a. This created pET-3aM with additional unique cloning sites for insertion of genes into the expression vector. Then, the Nde I site at the position of translation initiation was converted to an Nco I site using oligonucleotide-directed mutagenesis. The DNA sequence of pET-3aM in this region, 5'-CATATGG, was converted to 5'-CCCATGG in pBT430.

Plasmid DNA containing a cDNA may be appropriately digested to release a nucleic acid fragment encoding the protein. This fragment may then be purified on a 1% NuSieve GTG™ low melting agarose gel (FMC). Buffer and agarose contain 10 µg/ml ethidium bromide for visualization of the DNA fragment. The fragment can then be purified from the agarose gel by digestion with GELase™ (Epicentre Technologies) according to the manufacturer's instructions, ethanol precipitated, dried and resuspended in 20 µL of water. Appropriate oligonucleotide adapters may be ligated to the fragment using T4 DNA ligase (New England Biolabs, Beverly, MA). The fragment containing the ligated adapters can be purified from the excess adapters using low melting agarose as described above. The vector pBT430 is digested, dephosphorylated with alkaline phosphatase (NEB) and deproteinized with phenol/chloroform as described above. The prepared vector pBT430 and fragment can then be ligated at 16°C for 15 hours followed by transformation into DH5 electrocompetent cells (GIBCO BRL). Transformants can be selected on agar plates containing LB media and 100 µg/mL ampicillin. Transformants containing the gene encoding the instant polypeptides are then screened for the correct orientation with respect to the T7 promoter by restriction enzyme analysis.

For high level expression, a plasmid clone with the cDNA insert in the correct orientation relative to the T7 promoter can be transformed into *E. coli* strain BL21(DE3) (Studier et al. (1986) *J. Mol. Biol.* 189:113-130). Cultures are grown in

LB medium containing ampicillin (100 mg/L) at 25°C. At an optical density at 600 nm of approximately 1, IPTG (isopropylthio- $\beta$ -galactoside, the inducer) can be added to a final concentration of 0.4 mM and incubation can be continued for 3 h at 25°. Cells are then harvested by centrifugation and re-suspended in 50  $\mu$ L of 50 mM Tris-HCl at pH 8.0 containing 0.1 mM DTT and 0.2 mM phenyl methylsulfonyl fluoride. A small amount of 1 mm glass beads can be added and the mixture sonicated 3 times for about 5 seconds each time with a microprobe sonicator. The mixture is centrifuged and the protein concentration of the supernatant determined. One  $\mu$ g of protein from the soluble fraction of the culture can be separated by SDS-polyacrylamide gel electrophoresis. Gels can be observed for protein bands migrating at the expected molecular weight.

#### Expression of Maize Phytyl/prenyltransferase in Soybean Somatic Embryos

The ability to change the levels of total tocopherol levels in plants by transforming them with sequences encoding the maize phytyl/prenyltransferase was tested by preparing transgenic soybean somatic embryos and assaying the tocopherol and oil levels. Plasmid DNA from clone poo18chste82r was used as a template for the amplification of the open reading from pcr by using the following two primers AGC GCG GCC GCA TGG ACG CGC TTC GCC TAC GGC CGT(forward primer) and AGC GCG GCC GCT CAC CGC ACC AGA GGG ATG AGC AG(reverse primer). Pfu polymerase was used according to the manufacturers recommendations (Stratagene). The following pcr reaction mix contained the following: 5ng plasmid, 25nmoles dNTPs, 5% DMSO, 1x pcr buffer (supplied), 30nmoles primers, 5U pfu polymerase in 100ul reaction volume. The pcr reaction conditions were as follows: Step 1, 45s 94°C; step 2 25 cycles of 45s 94°C, 45s 58°C annealing, 2min extension 72°C. Step 3 72°C 10min, step 4 0°C. The pcr product was purified by agarose gel electrophoresis (1% agarose in TAE), the ethidium bromide visualized band cut out and purified from the gel by using a QIAquick Gel Extraction kit (Qiagen) according to the manufacturers recommendations. The purified pcr product (200ng) was ligated into the *srf*I site of the plasmid PCR-Script cloning vector and the resultant plasmid was used to transform E.coli DH10 cells. Colonies containing the 1.2kb NotI fragment were identified by antibiotic (ampicillin selection) and blue / white (IPTG + X-gal)

selection of colonies on LB/Amp plates. White (recombinant) colonies were picked and grown overnight on liquid LB/Amp culture. Positive clones were identified by plasmid preparation and restriction digest analysis for the presence of the 1.2kB *NotI* fragment. Positive clones were used as template to fully sequence the phytyl transferase orf (both strands). Plasmids containing the correct insert verified by nucleic acid sequence were digested with *NotI* and the 1.2kb fragment ligated to *NotI*-digested and phosphatase-treated pKS67. The plasmid pKS67 was prepared by replacing in pRB20 (described in U.S. Patent No. 5,846,784) the 800 bp Nos 3' fragment, with the 285 bp Nos 3' fragment containing the polyadenylation signal sequence and described in Depicker et al. (1982) *J. Mol. Appl. Genet.* 1:561-573. Clones were screened for the sense and antisense orientation of the phytyl/prenyltransferase insert fragment by restriction enzyme digestion.

#### *Transformation of Soybean Somatic Embryo Cultures*

The stock solutions and media shown in Table 4 were used for transformation and propagation of soybean somatic embryos:

Table 4 – Stock Solution and Media

Stock Solutions		Media	
<u>MS Sulfate 100x stock</u>		<u>SB55 (per Liter)</u>	
	(g/L)	10 mL of each MS stock	
MgSO <sub>4</sub> ·7H <sub>2</sub> O	37.0	1 mL of B5 Vitamin stock	
MnSO <sub>4</sub> ·H <sub>2</sub> O	1.69	0.8 g NH <sub>4</sub> NO <sub>3</sub>	
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.86	3.033 g KNO <sub>3</sub>	
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.0025	1 mL 2,4-D (10 mg/mL stock)	
<u>MS Halides 100x stock</u>		0.667 g asparagine	
CaCl <sub>2</sub> ·2H <sub>2</sub> O	44.0	pH 5.7	
KI	0.083	<u>SB103 (per Liter)</u>	
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.00125	1 pk. Murashige & Skoog salt mixture*	
KH <sub>2</sub> PO <sub>4</sub>	17.0	60 g maltose	
H <sub>3</sub> BO <sub>3</sub>	0.62	2 g gelrite	
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.025	pH 5.7	
Na <sub>2</sub> EDTA	3.724	<u>SB148 (per Liter)</u>	
FeSO <sub>4</sub> ·7H <sub>2</sub> O	2.784	1 pk. Murashige & Skoog salt mixture*	
<u>B5 Vitamin stock</u>		60 g maltose	
myo-inositol	100.0	1 mL B5 vitamin stock	
nicotinic acid	1.0	7 g agarose	
pyridoxine HCl	1.0	pH 5.7	
thiamine	10.0		

\*(Gibco BRL)

Soybean embryonic suspension cultures were maintained in 35 mL liquid media (SB55) on a rotary shaker (150 rpm) at 28°C with a mix of fluorescent and incandescent lights providing a 16 h day 8 h night cycle. Cultures were subcultured every 2 to 3 weeks by inoculating approximately 35 mg of tissue into 35 mL of fresh liquid media.

Soybean embryonic suspension cultures were transformed with the plasmid containing the phytyl/prenyltransferase sequence (positive orientation) by the method of particle gun bombardment (see Klein et al. (1987) *Nature* 327:70-73) using a DuPont Biolistic PDS1000/He instrument. Five µL of pKS93s plasmid DNA (1 g/L), 50 µL CaCl<sub>2</sub> (2.5 M), and 20 µL spermidine (0.1 M) were added to 50 µL of a 60 mg/mL 1 mm gold particle suspension. The particle preparation was

agitated for 3 minutes, spun on a microfuge for 10 seconds and the supernate removed. The DNA-coated particles were then washed once with 400  $\mu$ L of 70% ethanol and resuspended in 40  $\mu$ L of anhydrous ethanol. The DNA/particle suspension was sonicated three times for 1 second each. Five  $\mu$ L of the DNA-coated gold particles were then loaded on each macro carrier disk.

Approximately 300 to 400 mg of two-week-old suspension culture was placed in an empty 60 mm X 15 mm petri dish and the residual liquid removed from the tissue using a pipette. The tissue was placed about 3.5 inches away from the retaining screen and bombarded twice. Membrane rupture pressure was set at 1100 psi and the chamber was evacuated to -28 inches of Hg. Two plates were bombarded, and following bombardment, the tissue was divided in half, placed back into liquid media, and cultured as described above.

Fifteen days after bombardment, the liquid media was exchanged with fresh SB55 containing 50 mg/mL hygromycin. The selective media was refreshed weekly. Six weeks after bombardment, green, transformed tissue was isolated and inoculated into flasks to generate new transformed embryonic suspension cultures.

Transformed embryonic clusters were removed from liquid culture media and placed on a solid agar media, SB103, containing 0.5% charcoal to begin maturation. After 1 week, embryos were transferred to SB103 media minus charcoal. After 5 weeks on SB103 media, maturing embryos were separated and placed onto SB148 media. During maturation embryos were kept at 26°C with a mix of fluorescent and incandescent lights providing a 16 h day 8 h night cycle. After 3 weeks on SB148 media, embryos were analyzed for the expression of the tocopherols. Each embryonic cluster gave rise to 5 to 20 somatic embryos.

Non-transformed somatic embryos were cultured by the same method as used for the transformed somatic embryos.

### ***Analysis of Transformed Somatic Embryos***

At the end of 3 weeks on SB148 medium somatic embryos were harvested from 33 independently transformed lines. Pools of five embryos/event were pooled, the fresh weight noted, the embryos frozen on dry ice and lyophilized overnight. The corresponding dry weight was noted, the embryos pulverized with a glass rod and tocopherols and oil extracted by the addition of 0.5ml heptane (18h,

room temperature, dark). The embryos were re-extracted with 0.25ml of heptane the solutions pooled and centrifuged (5min, 12000g). The supernatant was stored in amber hplc autosampler vials at  $-20^{\circ}\text{C}$  prior to analysis.

HPLC analysis of the extracts was carried out using an HP1100 system (Agilent Technologies). 25ul of the heptane sample was applied to a Lichrosphere Si 60 column (5micron 4 x 12.5mm). The column was eluted with heptane/isopropanol (98:2 v/v) at a flow rate of 1ml/min. After 6minutes all four tocopherol isomers were eluted, as detected by a HP1100 fluorescence detector (excitation wavelength 295nm, emission wavelength 330nm). Individual tocopherol standards (Matreya) were diluted with HPLC grade heptane to levels between 1 and 200ng/ul to construct a six point external standard curve. Tocopherols in each sample were quantified using a standard curve run on the same day as the samples.

Total oil content of the samples was estimated by quantitative gas chromatography of the fatty acid methyl esters. 50ul samples were derivitized by addition to 0.5ml of a 1% (v/v) solution of sodium methoxide in methanol, 1 ug of undecanoic acid (17:0) dissolved in toluene was added as an internal standard. Derivitized fatty acids were extracted in 400ul heptane, fatty acids separated by glc and the peak heights quantitated by using a HP 6890 gas liquid chromatograph equipped with a fused silica capillary column 30m x i.d. 0.25mm coated with polar phase Omegawax 320 (Supelco In, Bellfonte,, PA), autosampler, flame ionization detector and ChemStation software on a HP

The example shown in Table 5 shows the data from 33 independent transformed lines of somatic soy embryos (five pooled embryos per line) transformed with KS67 containing the maize phytyl/prenyltransferase in the positive orientation. Normal ratios of tocopherol (ngT) / oil (ugOil) in somatic embryos are 2-5. Overexpression of the phytyl/prenyltransferase has increased the amount of tocopherol relative to oil. In particular in samples 16 and 17 the ng/ugOil ratios have doubled to be 10.9 and 10.1 respectively.

TABLE 5 – Lines Transformed with KS67 in Positive Orientation

Sample	Oil (mg)	Tocopherol (ng)	ngT/ugOil
1	313.2	1.25	3.98
2	162.5	0.51	3.2
3	195.9	1.29	6.6
4	133.7	0.69	5.2
5	323.5	0.95	2.9
6	18.6	0.13	7.1
7	121.3	0.32	2.6
8	98.9	0.73	7.4
9	175.2	0.5	2.8
10	314.5	1.3	4.1
11	99.4	0.5	5.1
12	75.1	0.23	3
13	105.9	0.59	5.5
14	381.2	1.15	3
15	248.1	1.44	5.8
16	103.8	1.13	10.9
17	165	1.67	10.1
18	117.3	0.5	4.3
19	255.7	0.77	3
20	365.1	1.8	4.9
21	253.9	0.79	31
22	88.7	0.59	6.6
23	454.2	1.23	2.7
24	352.5	1.61	4.6
25	240.9	0.63	2.6
26	404.2	2.19	5.4
27	323	1.52	4.7
28	386.2	2.28	5.9
29	253.5	1.28	5
30	211.9	1.35	6.4
31	460.5	1.3	2.8
32	161.7	1.19	7.3
33	275.5	1.66	6



Table 6 – Detailed Analysis of each of the Five Embryos in Transformed Lines 15 (control), 16, 17 and 18 (control).

Sample	Oil (mg)	Tocopherol (ng)	ngT/ugOil
SC5 15-1	1.36	10.6	7.8
SC5 15-2	0.75	7.2	9.7
SC5 15-3	0.93	5.4	5.8
SC5 15-4	6.67	37.04	5.6
SC5 15-5	1.35	10.1	7.5
SC5 16-1	0.8	15.8	19.7
SC5 16-2	0.4	10.7	26.8
SC5 16-3	4.21	27.5	6.5
SC5 16-4	0.2	3.5	17.5
SC5 16-5	2.7	35.7	13.2
SC5 17-1	0.4	44.3	111
SC5 17-2	0.2	39.6	200
SC5 17-3	5	58	11.7
SC5 17-4	1.29	11.6	9
SC5 17-5	24.7	197.8	13.5
SC5 18-1	32.1	43.4	1.4
SC5 18-2	31.6	6.1	1.9
SC5 18-3	2.99	11.6	3.9
SC5 18-4	0.7	6.1	8.7
SC5 18-5	0.5	3.4	7

The single embryo analysis in Table 6 was conducted to confirm the pooled embryo data provided in Table 5. It should also be noted that an alternative embodiment of the invention involves somatic soy embryos transformed with KS67 containing the maize phytyl/prenyltransferase in the reverse orientation.

The above examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims. All publications, patents, patent applications, and computer programs cited herein are hereby incorporated by reference.

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The present invention will be further described by reference to the following detailed examples. It is understood, however, that there are many extensions, variations, and modification on the basic theme of the present invention beyond that shown in the examples and description, which are within the spirit and scope of the present invention. All publications, patents, and patent application cited herein are hereby incorporated by reference.

## **WHAT IS CLAIMED IS**

1. An isolated nucleic acid comprising a member selected from the group consisting of:
  - (a) a polynucleotide that encodes a polypeptide of SEQ ID NO: 4, 12, 14, 16, 18, 20, 22, 24, 26 or 28;
  - (b) a polynucleotide amplified from a plant tissue nucleic acid library using the primers of SEQ ID NOS: 5-8, provided the polynucleotide is not SEQ ID NO: 9 or the genomic sequence of SEQ ID NO: 1 or 9.
  - (c) a polynucleotide comprising at least:
    - (i) 280 contiguous bases of SEQ ID NO:1,
    - (ii) 20 contiguous bases of SEQ ID NO: 3,
    - (iii) 30 contiguous bases of SEQ ID NO: 3;
    - (iv) 50 contiguous bases of SEQ ID NO: 11,
    - (v) 50 contiguous bases of SEQ ID NO: 13,
    - (vi) 297 contiguous bases of SEQ ID NO: 15,
    - (vii) 20 contiguous bases of the coding region of SEQ ID NO: 17, or
    - (viii) 30 contiguous bases of SEQ ID NO: 19, 21, 23, 25, 27 or 29;
  - (d) a polynucleotide encoding a plant or bacteria phytyl/prenyltransferase protein other than an *Arabidopsis thaliana* or *Synechocystis* phytyl/prenyltransferase protein;
  - (e) a polynucleotide having at least 50% sequence identity to SEQ ID NO: 3, wherein the % sequence identity is based on the entire coding sequence and is determined by BLAST 2.0 using default parameters;
  - (f) a polynucleotide having
    - (i) at least 70% sequence identity to SEQ ID NO: 3, 11, 13, 17, 19, 23, 25, 27 or 29,
    - (ii) at least 70% sequence identity to nucleotides spanning positions 226 to 1098 of SEQ ID NO: 15,
    - (iii) at least 72% sequence identity to SEQ ID NO: 21wherein the % sequence identity is based on the coding sequence and is determined by GAP using default parameters;

- (g) a polynucleotide having at least 90% sequence identity to SEQ ID NO: 1 wherein the % sequence identity is based on the entire sequence and is determined by GAP using default parameters;
  - (h) a polynucleotide which selectively hybridizes, under stringent hybridization conditions and a wash in 2X SSC at 50°C, to a hybridization probe the polynucleotide sequence of which consists of SEQ ID NO: 3, 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29, or the complement of SEQ ID NO: 3, 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29, provided the polynucleotide is not SEQ ID NO: 9, a genomic sequence of SEQ ID NO: 1 or 9, a nucleotide sequence of any length in the region between positions 55 to 365 of SEQ ID NO: 15 or a nucleotide sequence of any length in the region between positions 801 to 1159 of SEQ ID NO: 17;
  - (i) a polynucleotide comprising the sequence set forth in SEQ ID NO: 3, 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29;
  - (j) a polynucleotide consisting of the sequence set forth in SEQ ID NO: 1, and
  - (k) a polynucleotide complementary to a polynucleotide of (a) through (j).
2. The isolated nucleic acid of claim 1 wherein the polynucleotide of (c) further comprises contiguous nucleotides that encode for the first ten amino acids of SEQ ID NO: 4, 12, 14, 16, 18, 20, 22, 24, 26 or 28.
  3. The isolated nucleic acid of claim 1 wherein the phytyl/prenyltransferase polynucleotide of (d) is from maize, soybean, rice, wheat, *Arabidopsis thaliana* or *Synechocystis*.
  4. The isolated nucleic acid of claim 1 wherein the polynucleotide of (e) modulates a prenyllipid biosynthetic pathway.
  5. The isolated nucleic acid of claim 4 wherein 2-demethyl-phytylplastoquinol or 2-demethyl-plastoquinol-9 is modified.

6. The isolated nucleic acid of claim 1 wherein the polynucleotide of (f) modulates a prenyl lipid biosynthetic pathway.
7. The isolated nucleic acid of claim 6 wherein 2-demethyl-phytylplastoquinol or 2-demethyl-plastoquinol-9 is modified.
8. The isolated nucleic acid of claim 1 wherein the polynucleotide of (h) comprises at least 25 nucleotides in length and hybridizes under stringent conditions including a wash with 0.1X SSC at 60°C to a hybridization probe the polynucleotide sequence of which consists of SEQ ID NO: 3, 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29, or the complement of SEQ ID NO: 3, 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29, provided the polynucleotide is not SEQ ID NO: 9, a genomic sequence of SEQ ID NO: 1 or 9, a nucleotide sequence of any length in the region between positions 55 to 365 of SEQ ID NO: 15 or a nucleotide sequence of any length in the region between positions 801 to 1159 of SEQ ID NO: 17.
9. The isolated nucleic acid of claim 8 wherein the isolated nucleic acid modulates a prenyl lipid biosynthetic pathway.
10. The isolated nucleic acid of claim 9 wherein 2-demethyl-phytylplastoquinol or 2-demethyl-plastoquinol-9 is modified.
11. A vector comprising at least one nucleic acid of claim 1 or SEQ ID NO: 9.
12. An expression cassette comprising at least one nucleic acid of claim 1 or SEQ ID NO: 9 operably linked to a promoter, wherein the nucleic acid is in sense or antisense orientation.
13. A host cell into which is introduced with at least one expression cassette of claim 12.
14. The host cell of claim 13 that is a plant cell.

15. A transgenic plant comprising at least one expression cassette of claim 13.
16. The transgenic plant of claim 15, wherein the plant is corn, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley, millet, *Arabidopsis thaliana*, tomato, *Brassica*, vegetables, peppers, potatoes, apples, spinach, or lettuce.
17. A seed from the transgenic plant of claim 16.
18. The seed of claim 17, wherein the seed is from corn, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley, millet, *Arabidopsis thaliana*, tomato, *Brassica*, vegetables, peppers, potatoes, apples, spinach, or lettuce.
19. An isolated protein comprising a member selected from the group consisting of:
  - (a) a polypeptide comprising at least 25 contiguous amino acids of SEQ ID NO: 2, 4, 10, 12, 14, 16, 18, 20, 22, 24, 26 or 28;
  - (b) a polypeptide which is a plant or bacterial phytyl/prenyltransferase protein;
  - (c) a polypeptide comprising at least 55% sequence identity to SEQ ID NO: 2 or 4, wherein the % sequence identity is based on the entire sequence and is determined by BLAST 2.0 using default parameters and has at least one epitope in common with a phytyl/prenyltransferase;
  - (d) a polypeptide comprising at least
    - (i) 75% sequence identity to SEQ ID NO: 2, 4, 10, 12, 14, 16, 18, 20, 24, 26 or 28, or
    - (ii) 77% sequence identity to SEQ ID NO: 22, wherein the % sequence identity is based on the entire sequence and is determined by GAP using default parameters and has at least one epitope in common with a phytyl/prenyltransferase;
  - (e) a polypeptide encoded by a nucleic acid of SEQ ID NO: 1, 3, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29; and

(f) a polypeptide of SEQ ID NO: 2, 4, 10, 12, 14, 16, 18, 20, 22, 24, 26 or 28.

20. The protein of claim 19, wherein the polypeptide is catalytically active.
21. A ribonucleic acid sequence encoding the protein of claim 20.
22. A method for modulating the level of phytyl/prenyltransferase protein in a plant, comprising:
  - (a) stably transforming a plant cell with a phytyl/prenyltransferase polynucleotide operably linked to a promoter, wherein the polynucleotide is in sense or antisense orientation;
  - (b) growing the plant cell under plant growing conditions to produce a regenerated plant capable of expressing the polynucleotide for a time sufficient to modulate the level of phytyl/prenyltransferase protein in the plant.
23. The method of claim 22, wherein the phytyl/prenyltransferase polynucleotide is selected from those of SEQ ID NO: 1, 3, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29.
24. The method of claim 22, wherein the plant is corn, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley, millet or *Arabidopsis thaliana*, tomato, *Brassica*, vegetables, peppers, potatoes, apples, spinach, or lettuce.
25. The method of claim 22, wherein phytyl/prenyltransferase protein is increased.
26. The method of claim 22, wherein phytyl/prenyltransferase protein is decreased.
27. A method for modulating the level of tocopherol in a plant, comprising:



- (a) stably transforming a plant cell with a phytyl/prenyltransferase polynucleotide operably linked to a promoter, wherein the polynucleotide is in sense or antisense orientation;
- (b) growing the plant cell under plant growing conditions to produce a regenerated plant capable of expressing the polynucleotide for a time sufficient to modulate level of tocopherol in the plant.
28. The method of claim 27, wherein the phytyl/prenyltransferase polynucleotide is selected from SEQ ID NO 1, 3, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29.
29. A method for modulating the level of plastiquinone in a plant, comprising:
- (a) stably transforming a plant cell with a phytyl/prenyltransferase polynucleotide operably linked to a promoter, wherein the polynucleotide is in sense or antisense orientation;
- (b) growing the plant cell under plant growing conditions to produce a regenerated plant capable of expressing the polynucleotide for a time sufficient to modulate the level of plastiquinone in the plant.
30. The method of claim 29, wherein the phytyl/prenyltransferase polynucleotide is selected from SEQ ID NO: 1, 3, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29.

[illegible][illegible]

# SEQUENCE LISTING

<110> Della Penna, Dean  
Collakova, Eva  
Coughlan, Sean J.  
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<120> PHYTYL/PRENYLTRANSFERASE NUCLEIC ACIDS,  
POLYPEPTIDES AND USES THEREOF

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                                   Met Glu Ser
                                   1

ctg ctc tct agt tct tct ctt gtt tcc gct gct ggt ggg ttt tgt tgg      164
Leu Leu Ser Ser Ser Ser Leu Val Ser Ala Ala Gly Gly Phe Cys Trp
    5                10                15

aag aag cag aat cta aag ctc cac tct tta tca gaa atc cga gtt ctg      212
Lys Lys Gln Asn Leu Lys Leu His Ser Leu Ser Glu Ile Arg Val Leu
    20                25                30                35

cgt tgt gat tcg agt aaa gtt gtc gca aaa ccg aag ttt agg aac aat      260
Arg Cys Asp Ser Ser Lys Val Val Ala Lys Pro Lys Phe Arg Asn Asn
                40                45                50

ctt gtt agg cct gat ggt caa gga tct tca ttg ttg ttg tat cca aaa      308
Leu Val Arg Pro Asp Gly Gln Gly Ser Ser Leu Leu Leu Tyr Pro Lys
                55                60                65

cat aag tcg aga ttt cgg gtt aat gcc act gcg ggt cag cct gag gct      356
His Lys Ser Arg Phe Arg Val Asn Ala Thr Ala Gly Gln Pro Glu Ala
                70                75                80

ttc gac tcg aat agc aaa cag aag tct ttt aga gac tcg tta gat gcg      404
Phe Asp Ser Asn Ser Lys Gln Lys Ser Phe Arg Asp Ser Leu Asp Ala
    85                90                95

ttt tac agg ttt tct agg cct cat aca gtt att ggc aca gtg ctt agc      452

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Phe	Tyr	Arg	Phe	Ser	Arg	Pro	His	Thr	Val	Ile	Gly	Thr	Val	Leu	Ser		
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Ile	Leu	Ser	Val	Ser	Phe	Leu	Ala	Ala	Glu	Lys	Val	Ser	Asp	Ile	Ser		
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cct	tta	ctt	ttc	act	ggc	atc	ttg	gag	gct	gtt	gtt	gca	gct	ctc	atg		548
Pro	Leu	Leu	Phe	Thr	Gly	Ile	Leu	Glu	Ala	Val	Val	Ala	Ala	Leu	Met		
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Met	Asn	Ile	Tyr	Ile	Val	Gly	Leu	Asn	Gln	Leu	Ser	Asp	Val	Glu	Ile		
		150					155					160					
gat	aag	gtt	aac	aag	ccc	tat	ctt	cca	ttg	gca	tca	gga	gaa	tat	tct		644
Asp	Lys	Val	Asn	Lys	Pro	Tyr	Leu	Pro	Leu	Ala	Ser	Gly	Glu	Tyr	Ser		
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Val	Asn	Thr	Gly	Ile	Ala	Ile	Val	Ala	Ser	Phe	Ser	Ile	Met	Ser	Phe		
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Trp	Leu	Gly	Trp	Ile	Val	Gly	Ser	Trp	Pro	Leu	Phe	Trp	Ala	Leu	Phe		
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Val	Ser	Phe	Met	Leu	Gly	Thr	Ala	Tyr	Ser	Ile	Asn	Leu	Pro	Leu	Leu		
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cgg	tgg	aaa	aga	ttt	gca	ttg	gtt	gca	gca	atg	tgt	atc	ctc	gct	gtc		836
Arg	Trp	Lys	Arg	Phe	Ala	Leu	Val	Ala	Ala	Met	Cys	Ile	Leu	Ala	Val		
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cga	gct	att	att	gtt	caa	atc	gcc	ttt	tat	cta	cat	att	cag	aca	cat		884
Arg	Ala	Ile	Ile	Val	Gln	Ile	Ala	Phe	Tyr	Leu	His	Ile	Gln	Thr	His		
	245					250					255						
gtg	ttt	gga	aga	cca	atc	ttg	ttc	act	agg	cct	ctt	att	ttc	gcc	act		932
Val	Phe	Gly	Arg	Pro	Ile	Leu	Phe	Thr	Arg	Pro	Leu	Ile	Phe	Ala	Thr		
260					265				270					275			
gcg	ttt	atg	agc	ttt	ttc	tct	gtc	gtt	att	gca	ttg	ttt	aag	gat	ata		980
Ala	Phe	Met	Ser	Phe	Phe	Ser	Val	Val	Ile	Ala	Leu	Phe	Lys	Asp	Ile		
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cct	gat	atc	gaa	ggg	gat	aag	ata	ttc	gga	atc	cga	tca	ttc	tct	gta		1028
Pro	Asp	Ile	Glu	Gly	Asp	Lys	Ile	Phe	Gly	Ile	Arg	Ser	Phe	Ser	Val		
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Thr	Leu	Gly	Gln	Lys	Arg	Val	Phe	Trp	Thr	Cys	Val	Thr	Leu	Leu	Gln		
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Met	Ala	Tyr	Ala	Val	Ala	Ile	Leu	Val	Gly	Ala	Thr	Ser	Pro	Phe	Ile		
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tgg	agc	aaa	gtc	atc	tcg	gtt	gtg	ggg	cat	gtt	ata	ctc	gca	aca	act		1172
Trp	Ser	Lys	Val	Ile	Ser	Val	Val	Gly	His	Val	Ile	Leu	Ala	Thr	Thr		
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Leu Trp Ala Arg Ala Lys Ser Val Asp Leu Ser Ser Lys Thr Glu Ile	
360 365 370	

act tca tgt tat atg ttc ata tgg aag ctc ttt tat gca gag tac ttg	1268
Thr Ser Cys Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala Glu Tyr Leu	
375 380 385	

ctg tta cct ttt ttg aag tgactgacat tagaagagaa gaagatggag	1316
Leu Leu Pro Phe Leu Lys	
390	

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tgaactagtg aattagagtt ttattctgaa acatggcaga ctgcaaaaat atgtcaaaga	1436
tatgaatttc tgttgggtaa agaagtctct gcttgggcaa aatcttaagg ttcgggtgtgt	1496
tgatataatg ctaagcgaag aaatcgattc tatgtagaaa tttccgaaac tatgtgtaaa	1556
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Arg	Val	Leu	Arg	Cys	Asp	Ser	Ser	Lys	Val	Val	Ala	Lys	Pro	Lys	Phe		
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Arg	Asn	Asn	Leu	Val	Arg	Pro	Asp	Gly	Gln	Gly	Ser	Ser	Leu	Leu	Leu		
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Tyr	Pro	Lys	His	Lys	Ser	Arg	Phe	Arg	Val	Asn	Ala	Thr	Ala	Gly	Gln		
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Pro	Glu	Ala	Phe	Asp	Ser	Asn	Ser	Lys	Gln	Lys	Ser	Phe	Arg	Asp	Ser		
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Leu	Asp	Ala	Phe	Tyr	Arg	Phe	Ser	Arg	Pro	His	Thr	Val	Ile	Gly	Thr		
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Met	Ser	Phe	Trp	Leu	Gly	Trp	Ile	Val	Gly	Ser	Trp	Pro	Leu	Phe	Trp		
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Pro	Leu	Leu	Arg	Trp	Lys	Arg	Phe	Ala	Leu	Val	Ala	Ala	Met	Cys	Ile		
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Leu	Ala	Val	Arg	Ala	Ile	Ile	Val	Gln	Ile	Ala	Phe	Tyr	Leu	His	Ile		
			245					250					255				
Gln	Thr	His	Val	Phe	Gly	Arg	Pro	Ile	Leu	Phe	Thr	Arg	Pro	Leu	Ile		
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Phe	Ala	Thr	Ala	Phe	Met	Ser	Phe	Phe	Ser	Val	Val	Ile	Ala	Leu	Phe		
	275				280						285						
Lys	Asp	Ile	Pro	Asp	Ile	Glu	Gly	Asp	Lys	Ile	Phe	Gly	Ile	Arg	Ser		

290                      295                      300  
 Phe Ser Val Thr Leu Gly Gln Lys Arg Val Phe Trp Thr Cys Val Thr  
 305                      310                      315                      320  
 Leu Leu Gln Met Ala Tyr Ala Val Ala Ile Leu Val Gly Ala Thr Ser  
                     325                      330                      335  
 Pro Phe Ile Trp Ser Lys Val Ile Ser Val Val Gly His Val Ile Leu  
                     340                      345                      350  
 Ala Thr Thr Leu Trp Ala Arg Ala Lys Ser Val Asp Leu Ser Ser Lys  
                     355                      360                      365  
 Thr Glu Ile Thr Ser Cys Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala  
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 Glu Tyr Leu Leu Leu Pro Phe Leu Lys  
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 ccc gtg cgg ccc ggc gcg gcc cgc ccg cga gat cat ttt cta cca cca      101  
 Pro Val Arg Pro Gly Ala Ala Arg Pro Arg Asp His Phe Leu Pro Pro  
                     15                      20                      25  
  
 tgt tgt tcc ata caa cga aat ggt gaa gga cga att tgc ttt tct agc      149  
 Cys Cys Ser Ile Gln Arg Asn Gly Glu Gly Arg Ile Cys Phe Ser Ser  
                     30                      35                      40  
  
 caa agg acc caa ggt cct acc ttg cat cac cat cag aaa ttc ttc gaa      197  
 Gln Arg Thr Gln Gly Pro Thr Leu His His His Gln Lys Phe Phe Glu  
                     45                      50                      55  
  
 tgg aaa tcc tcc tat tgt agg ata tca cat cgg tca tta aat act tct      245  
 Trp Lys Ser Ser Tyr Cys Arg Ile Ser His Arg Ser Leu Asn Thr Ser  
                     60                      65                      70                      75  
  
 gtt aat gct tcg ggg caa cag ctg cag tct gaa cct gaa aca cat gat      293  
 Val Asn Ala Ser Gly Gln Gln Leu Gln Ser Glu Pro Glu Thr His Asp  
                     80                      85                      90  
  
 tct aca acc atc tgg agg gca ata tca tct tct cta gat gca ttt tac      341  
 Ser Thr Thr Ile Trp Arg Ala Ile Ser Ser Ser Leu Asp Ala Phe Tyr  
                     95                      100                      105  
  
 aga ttt tcc cgg cca cat act gtc ata gga aca gca tta agc ata gtc      389  
 Arg Phe Ser Arg Pro His Thr Val Ile Gly Thr Ala Leu Ser Ile Val  
                     110                      115                      120  
  
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 Ser Val Ser Leu Leu Ala Val Gln Ser Leu Ser Asp Ile Ser Pro Leu

125	130	135	
ttc ctc act ggt ttg ctg gag gca gtg gta gct gcc ctt ttc atg aat Phe Leu Thr Gly Leu Leu Glu Ala Val Val Ala Ala Leu Phe Met Asn 140 145 150 155			485
atc tat att gtt gga ctg aac cag tta ttc gac att gag ata gac aag Ile Tyr Ile Val Gly Leu Asn Gln Leu Phe Asp Ile Glu Ile Asp Lys 160 165 170			533
gtt aac aag cca act ctt cca ttg gca tct ggg gaa tac acc ctt gca Val Asn Lys Pro Thr Leu Pro Leu Ala Ser Gly Glu Tyr Thr Leu Ala 175 180 185			581
act ggg gtt gca ata gtt tcg gtc ttt gcc gct atg agc ttt ggc ctt Thr Gly Val Ala Ile Val Ser Val Phe Ala Ala Met Ser Phe Gly Leu 190 195 200			629
gga tgg gct gtt gga tca caa cct ctg ttt tgg gct ctt ttc ata agc Gly Trp Ala Val Gly Ser Gln Pro Leu Phe Trp Ala Leu Phe Ile Ser 205 210 215			677
ttt gtt ctt ggg act gca tat tca atc aat ctg ccg tac ctt cga tgg Phe Val Leu Gly Thr Ala Tyr Ser Ile Asn Leu Pro Tyr Leu Arg Trp 220 225 230 235			725
aag aga ttt gct gtt gtt gca gca ctg tgc ata tta gca gtt cgt gca Lys Arg Phe Ala Val Val Ala Ala Leu Cys Ile Leu Ala Val Arg Ala 240 245 250			773
gtg att gtt cag ctg gcc ttt ttt ctc cac att cag act ttt gtt ttc Val Ile Val Gln Leu Ala Phe Phe Leu His Ile Gln Thr Phe Val Phe 255 260 265			821
agg aga ccg gca gtg ttt tct agg cca tta tta ttt gca act gga ttt Arg Arg Pro Ala Val Phe Ser Arg Pro Leu Leu Phe Ala Thr Gly Phe 270 275 280			869
atg acg ttc ttc tct gtt gta ata gca cta ttc aag gat ata cct gac Met Thr Phe Phe Ser Val Val Ile Ala Leu Phe Lys Asp Ile Pro Asp 285 290 295			917
atc gaa ggg gac cgc ata ttc ggg atc cga tcc ttc agc gtc cgg tta Ile Glu Gly Asp Arg Ile Phe Gly Ile Arg Ser Phe Ser Val Arg Leu 300 305 310 315			965
ggg caa aag aag gtc ttt tgg atc tgc gtt ggc ttg ctt gag atg gcc Gly Gln Lys Lys Val Phe Trp Ile Cys Val Gly Leu Leu Glu Met Ala 320 325 330			1013
tac agc gtt gcg ata ctg atg gga gct acc tct tcc tgt ttg tgg agc Tyr Ser Val Ala Ile Leu Met Gly Ala Thr Ser Ser Cys Leu Trp Ser 335 340 345			1061
aaa aca gca acc atc gct ggc cat tcc ata ctt gcc gcg atc cta tgg Lys Thr Ala Thr Ile Ala Gly His Ser Ile Leu Ala Ala Ile Leu Trp 350 355 360			1109
agc tgc gcg cga tcg gtg gac ttg acg agc aaa gcc gca ata acg tcc Ser Cys Ala Arg Ser Val Asp Leu Thr Ser Lys Ala Ala Ile Thr Ser 365 370 375			1157

ttc tac atg ttc atc tgg aag ctg ttc tac gcg gag tac ctg ctc atc 1205  
 Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala Glu Tyr Leu Leu Ile  
 380 385 390 395

cct ctg gtg cgg tgagcgcgag gcgaggtggt ggcagacgga tcggcgtcgg 1257  
 Pro Leu Val Arg

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 35 40 45  
 Pro Thr Leu His His His Gln Lys Phe Phe Glu Trp Lys Ser Ser Tyr  
 50 55 60  
 Cys Arg Ile Ser His Arg Ser Leu Asn Thr Ser Val Asn Ala Ser Gly  
 65 70 75 80  
 Gln Gln Leu Gln Ser Glu Pro Glu Thr His Asp Ser Thr Thr Ile Trp  
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 Arg Ala Ile Ser Ser Ser Leu Asp Ala Phe Tyr Arg Phe Ser Arg Pro  
 100 105 110  
 His Thr Val Ile Gly Thr Ala Leu Ser Ile Val Ser Val Ser Leu Leu  
 115 120 125  
 Ala Val Gln Ser Leu Ser Asp Ile Ser Pro Leu Phe Leu Thr Gly Leu  
 130 135 140  
 Leu Glu Ala Val Val Ala Ala Leu Phe Met Asn Ile Tyr Ile Val Gly  
 145 150 155 160  
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 165 170 175  
 Leu Pro Leu Ala Ser Gly Glu Tyr Thr Leu Ala Thr Gly Val Ala Ile  
 180 185 190  
 Val Ser Val Phe Ala Ala Met Ser Phe Gly Leu Gly Trp Ala Val Gly  
 195 200 205  
 Ser Gln Pro Leu Phe Trp Ala Leu Phe Ile Ser Phe Val Leu Gly Thr  
 210 215 220  
 Ala Tyr Ser Ile Asn Leu Pro Tyr Leu Arg Trp Lys Arg Phe Ala Val  
 225 230 235 240  
 Val Ala Ala Leu Cys Ile Leu Ala Val Arg Ala Val Ile Val Gln Leu  
 245 250 255  
 Ala Phe Phe Leu His Ile Gln Thr Phe Val Phe Arg Arg Pro Ala Val  
 260 265 270  
 Phe Ser Arg Pro Leu Leu Phe Ala Thr Gly Phe Met Thr Phe Phe Ser  
 275 280 285  
 Val Val Ile Ala Leu Phe Lys Asp Ile Pro Asp Ile Glu Gly Asp Arg  
 290 295 300  
 Ile Phe Gly Ile Arg Ser Phe Ser Val Arg Leu Gly Gln Lys Lys Val  
 305 310 315 320  
 Phe Trp Ile Cys Val Gly Leu Leu Glu Met Ala Tyr Ser Val Ala Ile  
 325 330 335



Leu Met Gly Ala Thr Ser Ser Cys Leu Trp Ser Lys Thr Ala Thr Ile  
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 Ala Gly His Ser Ile Leu Ala Ala Ile Leu Trp Ser Cys Ala Arg Ser  
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 Val Asp Leu Thr Ser Lys Ala Ala Ile Thr Ser Phe Tyr Met Phe Ile  
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Ile Gly Thr Thr Leu Ser Val Trp Ala Val Tyr Leu Leu Thr Ile Leu	
20 25 30	
ggg gat gga aac tca gtt aac tcc cct gct tcc ctg gat tta gtg ttc	144
Gly Asp Gly Asn Ser Val Asn Ser Pro Ala Ser Leu Asp Leu Val Phe	
35 40 45	
ggc gct tgg ctg gcc tgc ctg ttg ggt aat gtg tac att gtc ggc ctc	192
Gly Ala Trp Leu Ala Cys Leu Leu Gly Asn Val Tyr Ile Val Gly Leu	
50 55 60	
aac caa ttg tgg gat gtg gac att gac cgc atc aat aag ccg aat ttg	240
Asn Gln Leu Trp Asp Val Asp Ile Asp Arg Ile Asn Lys Pro Asn Leu	
65 70 75 80	
ccc cta gct aac gga gat ttt tct atc gcc cag ggc cgt tgg att gtg	288
Pro Leu Ala Asn Gly Asp Phe Ser Ile Ala Gln Gly Arg Trp Ile Val	
85 90 95	
gga ctt tgt ggc gtt gct tcc ttg gcg atc gcc tgg gga tta ggg cta	336
Gly Leu Cys Gly Val Ala Ser Leu Ala Ile Ala Trp Gly Leu Gly Leu	
100 105 110	
tgg ctg ggg cta acg gtg ggc att agt ttg att att ggc acg gcc tat	384
Trp Leu Gly Leu Thr Val Gly Ile Ser Leu Ile Ile Gly Thr Ala Tyr	
115 120 125	
tcg gtg ccg cca gtg agg tta aag cgc ttt tcc ctg ctg gcg gcc ctg	432
Ser Val Pro Pro Val Arg Leu Lys Arg Phe Ser Leu Leu Ala Ala Leu	
130 135 140	
tgt att ctg acg gtg cgg gga att gtg gtt aac ttg ggc tta ttt tta	480
Cys Ile Leu Thr Val Arg Gly Ile Val Val Asn Leu Gly Leu Phe Leu	
145 150 155 160	
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Phe Phe Arg Ile Gly Leu Gly Tyr Pro Pro Thr Leu Ile Thr Pro Ile	
165 170 175	
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Trp Val Leu Thr Leu Phe Ile Leu Val Phe Thr Val Ala Ile Ala Ile	
180 185 190	
ttt aaa gat gtg cca gat atg gaa ggc gat cgg caa ttt aag att caa	624
Phe Lys Asp Val Pro Asp Met Glu Gly Asp Arg Gln Phe Lys Ile Gln	
195 200 205	
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Thr Leu Thr Leu Gln Ile Gly Lys Gln Asn Val Phe Arg Gly Thr Leu	
210 215 220	
att tta ctc act ggt tgt tat tta gcc atg gca atc tgg ggc tta tgg	720
Ile Leu Leu Thr Gly Cys Tyr Leu Ala Met Ala Ile Trp Gly Leu Trp	
225 230 235 240	

gcg gct atg cct tta aat act gct ttc ttg att gtt tcc cat ttg tgc 768  
Ala Ala Met Pro Leu Asn Thr Ala Phe Leu Ile Val Ser His Leu Cys  
245 250 255

tta tta gcc tta ctc tgg tgg cgg agt cga gat gta cac tta gaa agc 816  
Leu Leu Ala Leu Leu Trp Trp Arg Ser Arg Asp Val His Leu Glu Ser  
260 265 270

aaa acc gaa att gct agt ttt tat cag ttt att tgg aag cta ttt ttc 864  
Lys Thr Glu Ile Ala Ser Phe Tyr Gln Phe Ile Trp Lys Leu Phe Phe  
275 280 285

tta gag tac ttg ctg tat ccc ttg gct ctg tgg tta cct aat ttt tct 912  
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Asn Thr Ile Phe \*  
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Gly Asp Gly Asn Ser Val Asn Ser Pro Ala Ser Leu Asp Leu Val Phe  
35 40 45  
Gly Ala Trp Leu Ala Cys Leu Leu Gly Asn Val Tyr Ile Val Gly Leu  
50 55 60  
Asn Gln Leu Trp Asp Val Asp Ile Asp Arg Ile Asn Lys Pro Asn Leu  
65 70 75 80  
Pro Leu Ala Asn Gly Asp Phe Ser Ile Ala Gln Gly Arg Trp Ile Val  
85 90 95  
Gly Leu Cys Gly Val Ala Ser Leu Ala Ile Ala Trp Gly Leu Gly Leu  
100 105 110  
Trp Leu Gly Leu Thr Val Gly Ile Ser Leu Ile Ile Gly Thr Ala Tyr  
115 120 125  
Ser Val Pro Pro Val Arg Leu Lys Arg Phe Ser Leu Leu Ala Ala Leu  
130 135 140  
Cys Ile Leu Thr Val Arg Gly Ile Val Val Asn Leu Gly Leu Phe Leu  
145 150 155 160  
Phe Phe Arg Ile Gly Leu Gly Tyr Pro Pro Thr Leu Ile Thr Pro Ile  
165 170 175  
Trp Val Leu Thr Leu Phe Ile Leu Val Phe Thr Val Ala Ile Ala Ile  
180 185 190  
Phe Lys Asp Val Pro Asp Met Glu Gly Asp Arg Gln Phe Lys Ile Gln  
195 200 205  
Thr Leu Thr Leu Gln Ile Gly Lys Gln Asn Val Phe Arg Gly Thr Leu  
210 215 220  
Ile Leu Leu Thr Gly Cys Tyr Leu Ala Met Ala Ile Trp Gly Leu Trp  
225 230 235 240  
Ala Ala Met Pro Leu Asn Thr Ala Phe Leu Ile Val Ser His Leu Cys  
245 250 255  
Leu Leu Ala Leu Leu Trp Trp Arg Ser Arg Asp Val His Leu Glu Ser  
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[illegible]

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atg aac gtt tat gta gta ggg ctg aac aag gtc aat aag cca acc ctc 213  
Met Asn Val Tyr Val Val Gly Leu Asn Lys Val Asn Lys Pro Thr Leu  
25 30 35

cca tta tcg ttc gga gag ttt tca atg cca act gca gta ttg tta gta 261  
Pro Leu Ser Phe Gly Glu Phe Ser Met Pro Thr Ala Val Leu Leu Val  
40 45 50

gtg gca ttc ttg gtc atg agc att agc atc gga ata aga tca aag tct 309  
Val Ala Phe Leu Val Met Ser Ile Ser Ile Gly Ile Arg Ser Lys Ser  
55 60 65

gct cca ttg atg tgt gct ttg ctt gtt tgc ttc ctt ctt gga agc gca 357  
Ala Pro Leu Met Cys Ala Leu Leu Val Cys Phe Leu Leu Gly Ser Ala  
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Tyr	Pro	Ile	Asp	Val	Pro	Leu	Leu	Arg	Trp	Lys	Arg	His	Ala	Phe	Leu	
85					90					95					100	

gct gca ttc tgc ata atc ttt gtg agg cct gta gtg gtc cag tta gct 453  
Ala Ala Phe Cys Ile Ile Phe Val Arg Pro Val Val Val Gln Leu Ala  
105 110 115

ttc ttt gca cac atg cag caa cat gtt ctg aag agg ccc ttg gca cct 501  
Phe Phe Ala His Met Gln Gln His Val Leu Lys Arg Pro Leu Ala Pro  
120 125 130

aca agg tcg gtg gtc ttt gca aca tgt ttc atg tgt tgc ttc gct gca 549  
Thr Arg Ser Val Val Phe Ala Thr Cys Phe Met Cys Cys Phe Ala Ala  
135 140 145

gta ata gcg cta ttc aag gat att cct gat gtc gat gga gat aga gat 597  
Val Ile Ala Leu Phe Lys Asp Ile Pro Asp Val Asp Gly Asp Arg Asp  
150 155 160

ttc ggc att cag tcc atg act gta cga tta ggc caa cag aga gtg cat 645

Phe	Gly	Ile	Gln	Ser	Met	Thr	Val	Arg	Leu	Gly	Gln	Gln	Arg	Val	His	
165					170					175					180	
agg	ctc	tgc	att	aat	att	ctc	atg	aca	gca	tac	gca	gcc	gca	att	ttg	693
Arg	Leu	Cys	Ile	Asn	Ile	Leu	Met	Thr	Ala	Tyr	Ala	Ala	Ala	Ile	Leu	
				185					190					195		
gta	ggc	gcg	tca	tct	acg	aac	ctg	tat	cag	aag	att	gtc	att	gtg	tct	741
Val	Gly	Ala	Ser	Ser	Thr	Asn	Leu	Tyr	Gln	Lys	Ile	Val	Ile	Val	Ser	
			200					205					210			
ggt	cat	ggc	ttg	ctt	gcc	tcc	aca	ctc	tgg	caa	aga	gca	caa	caa	ttt	789
Gly	His	Gly	Leu	Leu	Ala	Ser	Thr	Leu	Trp	Gln	Arg	Ala	Gln	Gln	Phe	
		215					220					225				
gac	att	gag	aat	aag	gat	tgt	atc	aca	caa	ttt	tat	atg	ttc	att	tgg	837
Asp	Ile	Glu	Asn	Lys	Asp	Cys	Ile	Thr	Gln	Phe	Tyr	Met	Phe	Ile	Trp	
		230				235					240					
aag	tta	ttc	tac	gcc	gag	tat	ttt	ctt	ata	cca	ttt	gtg	tag			879
Lys	Leu	Phe	Tyr	Ala	Glu	Tyr	Phe	Leu	Ile	Pro	Phe	Val	*			
		245				250				255						
taaagaatca	tgcaagaac	aacacccctg	ctatagacat	gtgaagggttt	attgctaattg											939
ttactctacc	ccctgctata	gacatgtgaa	ggttttattgc	taatgttact	ctaccgaatg											999
gtctgaatgt	ctatgcgtca	tttgaatgta	atatgactat	ttgttgatc	agggttaacaa											1059
ctggagcaaa	tgtacatgt	atattaagca	ttaatttaac	tgcattcattt	gtaccatgta											1119
tattatgact	atgtatgaga	tattgtctct	tattagtact	ggatgtgatg	tgtcttatta											1179
tgactatgga	tgagactttt	gtgatgtaat	tgatgagact	atgggttttaa	atattgttat											1239
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 <212> PRT  
 <213> Zea mays

<400> 12

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Lys	Pro	Thr	Leu	Pro	Leu	Ser	Phe	Gly	Glu	Phe	Ser	Met	Pro	Thr	Ala	
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Val	Leu	Leu	Val	Val	Ala	Phe	Leu	Val	Met	Ser	Ile	Ser	Ile	Gly	Ile	
		50			55						60					
Arg	Ser	Lys	Ser	Ala	Pro	Leu	Met	Cys	Ala	Leu	Leu	Val	Cys	Phe	Leu	
65				70					75						80	
Leu	Gly	Ser	Ala	Tyr	Pro	Ile	Asp	Val	Pro	Leu	Leu	Arg	Trp	Lys	Arg	
			85					90						95		
His	Ala	Phe	Leu	Ala	Ala	Phe	Cys	Ile	Ile	Phe	Val	Arg	Pro	Val	Val	
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Val	Gln	Leu	Ala	Phe	Phe	Ala	His	Met	Gln	Gln	His	Val	Leu	Lys	Arg	
			115				120					125				
Pro	Leu	Ala	Pro	Thr	Arg	Ser	Val	Val	Phe	Ala	Thr	Cys	Phe	Met	Cys	
			130			135						140				
Cys	Phe	Ala	Ala	Val	Ile	Ala	Leu	Phe	Lys	Asp	Ile	Pro	Asp	Val	Asp	
145				150					155						160	
Gly	Asp	Arg	Asp	Phe	Gly	Ile	Gln	Ser	Met	Thr	Val	Arg	Leu	Gly	Gln	
			165					170						175		
Gln	Arg	Val	His	Arg	Leu	Cys	Ile	Asn	Ile	Leu	Met	Thr	Ala	Tyr	Ala	
			180					185					190			
Ala	Ala	Ile	Leu	Val	Gly	Ala	Ser	Ser	Thr	Asn	Leu	Tyr	Gln	Lys	Ile	

	195		200		205
Val	Ile Val Ser Gly His Gly Leu Leu Ala Ser Thr Leu Trp Gln Arg				
	210		215		220
Ala	Gln Gln Phe Asp Ile Glu Asn Lys Asp Cys Ile Thr Gln Phe Tyr				
225		230		235	240
Met	Phe Ile Trp Lys Leu Phe Tyr Ala Glu Tyr Phe Leu Ile Pro Phe				
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Val					

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Ala Arg Phe Leu Ala Ala Pro Ala Ile Arg Val Ile Ser Pro Ser Arg	
20 25 30	
ccc gcg ctg ccg ctc ctc tca tcc gcc tcc gca ggc ggc ttc cct cac	144
Pro Ala Leu Pro Leu Leu Ser Ser Ala Ser Ala Gly Gly Phe Pro His	
35 40 45	
gcc tct cgc gct ccc tgc agt gcc gcc cgc gag cac cgc cgc ggc acc	192
Ala Ser Arg Ala Pro Cys Ser Ala Ala Arg Glu His Arg Arg Gly Thr	
50 55 60	
gtg cgg gaa tgc tct cga gct gat gct gct gga gca gct cca tta tca	240
Val Arg Glu Cys Ser Arg Ala Asp Ala Ala Gly Ala Ala Pro Leu Ser	
65 70 75 80	
aag aca ctg tta gac ctc aag gat tcc tgc tgg aga ttt tta agg cca	288
Lys Thr Leu Leu Asp Leu Lys Asp Ser Cys Trp Arg Phe Leu Arg Pro	
85 90 95	
cat aca atc cga gga act gct tta gga tcc ata gca ttg gtt gcg aga	336
His Thr Ile Arg Gly Thr Ala Leu Gly Ser Ile Ala Leu Val Ala Arg	
100 105 110	
gcc ttg ata gag aat tcc cat ctg ata aac tgg tgg ttg ata ttc aaa	384
Ala Leu Ile Glu Asn Ser His Leu Ile Asn Trp Trp Leu Ile Phe Lys	
115 120 125	
gca ttc tat gga ctt ggg gca ttg ata ttt ggc aat ggt tac ata gtt	432
Ala Phe Tyr Gly Leu Gly Ala Leu Ile Phe Gly Asn Gly Tyr Ile Val	
130 135 140	
ggg att aat cag atc tat gat gtt gct att gac aag gta aac aag cca	480
Gly Ile Asn Gln Ile Tyr Asp Val Ala Ile Asp Lys Val Asn Lys Pro	
145 150 155 160	
tat tta ccc att gct gct ggt gat ctc tca att cag tca gca tgg ttg	528
Tyr Leu Pro Ile Ala Ala Gly Asp Leu Ser Ile Gln Ser Ala Trp Leu	

165																170						175						
ttg	gtg	ata	tta	ttt	gca	gct	gca	ggt	ttt	tca	att	gtt	ata	tca	aac	576												
Leu	Val	Ile	Leu	Phe	Ala	Ala	Ala	Gly	Phe	Ser	Ile	Val	Ile	Ser	Asn													
			180				185						190															
ttt	gga	cct	ttc	att	acc	tct	cta	tac	tgc	ctt	ggc	cta	ttt	ctt	ggc	624												
Phe	Gly	Pro	Phe	Ile	Thr	Ser	Leu	Tyr	Cys	Leu	Gly	Leu	Phe	Leu	Gly													
			195				200						205															
act	ata	tat	tct	gtt	cct	cca	ttt	aga	ctg	aag	aga	tat	ccg	gtt	gct	672												
Thr	Ile	Tyr	Ser	Val	Pro	Pro	Phe	Arg	Leu	Lys	Arg	Tyr	Pro	Val	Ala													
			210				215						220															
gct	ttt	ctt	atc	att	gca	acg	gtt	cgt	ggc	ttc	ctt	ctc	aac	ttt	ggc	720												
Ala	Phe	Leu	Ile	Ile	Ala	Thr	Val	Arg	Gly	Phe	Leu	Leu	Asn	Phe	Gly													
225				230						235			240															
gtg	tac	tat	gct	act	agg	gct	gca	cta	ggt	ctt	aca	ttc	caa	tgg	agc	768												
Val	Tyr	Tyr	Ala	Thr	Arg	Ala	Ala	Leu	Gly	Leu	Thr	Phe	Gln	Trp	Ser													
			245				250						255															
tcc	cct	gtt	gct	ttc	att	aca	tgc	ttc	gtg	aca	cta	ttt	gct	ttg	gtc	816												
Ser	Pro	Val	Ala	Phe	Ile	Thr	Cys	Phe	Val	Thr	Leu	Phe	Ala	Leu	Val													
			260				265						270															
att	gct	ata	acc	aaa	gat	ctc	cct	gat	gtt	gaa	gga	gat	cgc	aag	tat	864												
Ile	Ala	Ile	Thr	Lys	Asp	Leu	Pro	Asp	Val	Glu	Gly	Asp	Arg	Lys	Tyr													
			275				280						285															
caa	ata	tca	act	ttg	gca	aca	aag	ctt	ggt	gtc	aga	aat	att	gca	ttc	912												
Gln	Ile	Ser	Thr	Leu	Ala	Thr	Lys	Leu	Gly	Val	Arg	Asn	Ile	Ala	Phe													
			290				295						300															
ctt	gga	tct	ggc	tta	tta	tta	gca	aac	tat	att	gct	gct	att	gct	gta	960												
Leu	Gly	Ser	Gly	Leu	Leu	Leu	Ala	Asn	Tyr	Ile	Ala	Ala	Ile	Ala	Val													
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gct	ttt	acc	atg	cct	cag	gat	ttc	agg	tgc	act	gta	atg	gtt	cct	gtg	1008												
Ala	Phe	Thr	Met	Pro	Gln	Asp	Phe	Arg	Cys	Thr	Val	Met	Val	Pro	Val													
			325				330						335															
cat	gct	gtc	ctt	gct	ggc	ggc	tta	att	ttc	cag	aca	tgg	gtt	ctg	gag	1056												
His	Ala	Val	Leu	Ala	Gly	Gly	Leu	Ile	Phe	Gln	Thr	Trp	Val	Leu	Glu													
			340				345						350															
caa	gcg	aag	tac	aga	aag	gat	gct	att	tcg	cag	tac	tat	cgg	ttc	ata	1104												
Gln	Ala	Lys	Tyr	Arg	Lys	Asp	Ala	Ile	Ser	Gln	Tyr	Tyr	Arg	Phe	Ile													
			355				360						365															
tgg	aat	ctc	ttc	tat	gct	gaa	tat	atc	ttc	ttc	ccg	tta	ata	tag		1149												
Trp	Asn	Leu	Phe	Tyr	Ala	Glu	Tyr	Ile	Phe	Phe	Pro	Leu	Ile	*														
			370				375						380															
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gtgtcgcggt	ctgtacggtc	ttact																										

tagtaggaac ctcaactttt accccatoga ttggaagact gaaacttctt gcatgcgat 1689  
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 <211> 382  
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 <213> Zea mays

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 35 40 45  
 Ala Ser Arg Ala Pro Cys Ser Ala Ala Arg Glu His Arg Arg Gly Thr  
 50 55 60  
 Val Arg Glu Cys Ser Arg Ala Asp Ala Ala Gly Ala Ala Pro Leu Ser  
 65 70 75 80  
 Lys Thr Leu Leu Asp Leu Lys Asp Ser Cys Trp Arg Phe Leu Arg Pro  
 85 90 95  
 His Thr Ile Arg Gly Thr Ala Leu Gly Ser Ile Ala Leu Val Ala Arg  
 100 105 110  
 Ala Leu Ile Glu Asn Ser His Leu Ile Asn Trp Trp Leu Ile Phe Lys  
 115 120 125  
 Ala Phe Tyr Gly Leu Gly Ala Leu Ile Phe Gly Asn Gly Tyr Ile Val  
 130 135 140  
 Gly Ile Asn Gln Ile Tyr Asp Val Ala Ile Asp Lys Val Asn Lys Pro  
 145 150 155 160  
 Tyr Leu Pro Ile Ala Ala Gly Asp Leu Ser Ile Gln Ser Ala Trp Leu  
 165 170 175  
 Leu Val Ile Leu Phe Ala Ala Ala Gly Phe Ser Ile Val Ile Ser Asn  
 180 185 190  
 Phe Gly Pro Phe Ile Thr Ser Leu Tyr Cys Leu Gly Leu Phe Leu Gly  
 195 200 205  
 Thr Ile Tyr Ser Val Pro Pro Phe Arg Leu Lys Arg Tyr Pro Val Ala  
 210 215 220  
 Ala Phe Leu Ile Ile Ala Thr Val Arg Gly Phe Leu Leu Asn Phe Gly  
 225 230 235 240  
 Val Tyr Tyr Ala Thr Arg Ala Ala Leu Gly Leu Thr Phe Gln Trp Ser  
 245 250 255  
 Ser Pro Val Ala Phe Ile Thr Cys Phe Val Thr Leu Phe Ala Leu Val  
 260 265 270  
 Ile Ala Ile Thr Lys Asp Leu Pro Asp Val Glu Gly Asp Arg Lys Tyr  
 275 280 285  
 Gln Ile Ser Thr Leu Ala Thr Lys Leu Gly Val Arg Asn Ile Ala Phe  
 290 295 300  
 Leu Gly Ser Gly Leu Leu Leu Ala Asn Tyr Ile Ala Ala Ile Ala Val  
 305 310 315 320  
 Ala Phe Thr Met Pro Gln Asp Phe Arg Cys Thr Val Met Val Pro Val  
 325 330 335  
 His Ala Val Leu Ala Gly Gly Leu Ile Phe Gln Thr Trp Val Leu Glu  
 340 345 350  
 Gln Ala Lys Tyr Arg Lys Asp Ala Ile Ser Gln Tyr Tyr Arg Phe Ile  
 355 360 365  
 Trp Asn Leu Phe Tyr Ala Glu Tyr Ile Phe Phe Pro Leu Ile  
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<213> Oryza sativa

<220>

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Met Asp Ser Leu Arg Leu Arg Pro Ser Leu Leu Ala Ala Arg Ala Pro	
1 5 10 15	
ggc gcg gcc tcg ctg ccg cct ctc cgg cga gat cac ttt cta cca cct	154
Gly Ala Ala Ser Leu Pro Pro Leu Arg Arg Asp His Phe Leu Pro Pro	
20 25 30	
tta tgt tct atc cat aga aat ggt aaa cgg cca gtt tct ttg tcc agc	202
Leu Cys Ser Ile His Arg Asn Gly Lys Arg Pro Val Ser Leu Ser Ser	
35 40 45	
caa agg acc caa ggt cct tcc ttc gat caa tgt cag aaa ttc ttt ggt	250
Gln Arg Thr Gln Gly Pro Ser Phe Asp Gln Cys Gln Lys Phe Phe Gly	
50 55 60	
tgg aaa tcc tcc cac cac agg ata cca cat cga cca aca tct agt tcc	298
Trp Lys Ser Ser His His Arg Ile Pro His Arg Pro Thr Ser Ser Ser	
65 70 75 80	
gct gac gct tcg gga caa cct cta caa tct tca gct gaa gca cat gat	346
Ala Asp Ala Ser Gly Gln Pro Leu Gln Ser Ser Ala Glu Ala His Asp	
85 90 95	
tca tca agt ata tgg aag cca ata tca tct tct ccg gat gca ttt tac	394
Ser Ser Ser Ile Trp Lys Pro Ile Ser Ser Ser Pro Asp Ala Phe Tyr	
100 105 110	
agg ttt tct egg cca cat act gtc ata gga aca gca ctt agc ata gtc	442
Arg Phe Ser Arg Pro His Thr Val Ile Gly Thr Ala Leu Ser Ile Val	
115 120 125	
tca gtt tcg ctg cta gct gtt gag aat ttg tcc gat gtg tct ccc ttg	490
Ser Val Ser Leu Leu Ala Val Glu Asn Leu Ser Asp Val Ser Pro Leu	
130 135 140	
ttc ctc act ggt ttg ctg gag gca gtg gta gca gct ctt ttc atg aac	538
Phe Leu Thr Gly Leu Leu Glu Ala Val Val Ala Ala Leu Phe Met Asn	
145 150 155 160	
atc tat atc gtt gga ttg aat cag ttg ttc gac att gag ata gat aag	586
Ile Tyr Ile Val Gly Leu Asn Gln Leu Phe Asp Ile Glu Ile Asp Lys	
165 170 175	
gtt aac aag cca act ctt cca tta gca tct ggg gaa tat tct cct gca	634
Val Asn Lys Pro Thr Leu Pro Leu Ala Ser Gly Glu Tyr Ser Pro Ala	
180 185 190	
act gga gtt gca ctt gta tca gcc ttc gct gct atg agc ttt ggc ctt	682
Thr Gly Val Ala Leu Val Ser Ala Phe Ala Ala Met Ser Phe Gly Leu	
195 200 205	
gga tgg gct gtt gga tca cag cct ctg ttc ctg gct ctt ttc att agc	730
Gly Trp Ala Val Gly Ser Gln Pro Leu Phe Leu Ala Leu Phe Ile Ser	

210	215	220	
ttt att ctt gga aca gca tat tgc att aat ctg cca ttc ctg aga tgg			778
Phe Ile Leu Gly Thr Ala Tyr Ser Ile Asn Leu Pro Phe Leu Arg Trp			
225	230	235	240
aag aga tct gct gtt gtt gca gca ctt tgc ata tta gca gtc cgt gca			826
Lys Arg Ser Ala Val Val Ala Ala Leu Cys Ile Leu Ala Val Arg Ala			
	245	250	255
gtg att gtt cag ctg gca ttt ttt ctc cac att cag aca ttc gta ttc			874
Val Ile Val Gln Leu Ala Phe Phe Leu His Ile Gln Thr Phe Val Phe			
	260	265	270
aga aga cca gca gtc ttt acc agg cca ttg att ttt gca act gca ttc			922
Arg Arg Pro Ala Val Phe Thr Arg Pro Leu Ile Phe Ala Thr Ala Phe			
	275	280	285
atg acc ttt ttc tcc gtt gta ata gca ttg ttc aag gat ata cct gat			970
Met Thr Phe Phe Ser Val Val Ile Ala Leu Phe Lys Asp Ile Pro Asp			
	290	295	300
att gaa gga gac cgt att ttt ggt atc aaa tct ttc agt gtt cga tta			1018
Ile Glu Gly Asp Arg Ile Phe Gly Ile Lys Ser Phe Ser Val Arg Leu			
	305	310	320
ggt caa aag aag gtt ttc tgg att tgt gtt ggt ctg ctc gag atg gct			1066
Gly Gln Lys Lys Val Phe Trp Ile Cys Val Gly Leu Leu Glu Met Ala			
	325	330	335
tat tgt gtt gca ata ttg atg gga gct act tct gcc tgt ttg tgg agc			1114
Tyr Cys Val Ala Ile Leu Met Gly Ala Thr Ser Ala Cys Leu Trp Ser			
	340	345	350
aaa tac gca act gtg gtg gga cat gca atc ctt gcg gca atc cta tgg			1162
Lys Tyr Ala Thr Val Val Gly His Ala Ile Leu Ala Ala Ile Leu Trp			
	355	360	365
aac cgc tca cgg tgc att gat ctg aca agc aaa act gca atc act tct			1210
Asn Arg Ser Arg Ser Ile Asp Leu Thr Ser Lys Thr Ala Ile Thr Ser			
	370	375	380
ttc tac atg ttt atc tgg aag ctg ttc tac gcg gaa tac ctt ctc att			1258
Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala Glu Tyr Leu Leu Ile			
	385	390	400
cct ctt gta agg tga caaaggcgat tactccaggt agattggaat tggatcatgg			1313
Pro Leu Val Arg *			
ctggatggat gaacggacgg cgccccataa aatcacctgc aaatcaccgc gtacacatgt			1373
tgacatcctg catccagata tgatattgat agatcatcgt cggcaccatc attcctctga			1433
aagatttcgc acggcatttc aacctccaac tcccaacgta cccccaaaaa agtaactagg			1493
ccaggtgagc atctgctagc ctatagtaga cgttattgga acagtggtag tacttggttag			1553
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aaaaa			1618
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<213> Oryza sativa			

<400> 16  
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Gly Ala Ala Ser Leu Pro Pro Leu Arg Arg Asp His Phe Leu Pro Pro  
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Leu Cys Ser Ile His Arg Asn Gly Lys Arg Pro Val Ser Leu Ser Ser  
35 40 45  
Gln Arg Thr Gln Gly Pro Ser Phe Asp Gln Cys Gln Lys Phe Phe Gly  
50 55 60  
Trp Lys Ser Ser His His Arg Ile Pro His Arg Pro Thr Ser Ser Ser  
65 70 75 80  
Ala Asp Ala Ser Gly Gln Pro Leu Gln Ser Ser Ala Glu Ala His Asp  
85 90 95  
Ser Ser Ser Ile Trp Lys Pro Ile Ser Ser Ser Pro Asp Ala Phe Tyr  
100 105 110  
Arg Phe Ser Arg Pro His Thr Val Ile Gly Thr Ala Leu Ser Ile Val  
115 120 125  
Ser Val Ser Leu Leu Ala Val Glu Asn Leu Ser Asp Val Ser Pro Leu  
130 135 140  
Phe Leu Thr Gly Leu Leu Glu Ala Val Val Ala Ala Leu Phe Met Asn  
145 150 155 160  
Ile Tyr Ile Val Gly Leu Asn Gln Leu Phe Asp Ile Glu Ile Asp Lys  
165 170 175  
Val Asn Lys Pro Thr Leu Pro Leu Ala Ser Gly Glu Tyr Ser Pro Ala  
180 185 190  
Thr Gly Val Ala Leu Val Ser Ala Phe Ala Ala Met Ser Phe Gly Leu  
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Gly Trp Ala Val Gly Ser Gln Pro Leu Phe Leu Ala Leu Phe Ile Ser  
210 215 220  
Phe Ile Leu Gly Thr Ala Tyr Ser Ile Asn Leu Pro Phe Leu Arg Trp  
225 230 235 240  
Lys Arg Ser Ala Val Val Ala Ala Leu Cys Ile Leu Ala Val Arg Ala  
245 250 255  
Val Ile Val Gln Leu Ala Phe Phe Leu His Ile Gln Thr Phe Val Phe  
260 265 270  
Arg Arg Pro Ala Val Phe Thr Arg Pro Leu Ile Phe Ala Thr Ala Phe  
275 280 285  
Met Thr Phe Phe Ser Val Val Ile Ala Leu Phe Lys Asp Ile Pro Asp  
290 295 300  
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305 310 315 320  
Gly Gln Lys Lys Val Phe Trp Ile Cys Val Gly Leu Leu Glu Met Ala  
325 330 335  
Tyr Cys Val Ala Ile Leu Met Gly Ala Thr Ser Ala Cys Leu Trp Ser  
340 345 350  
Lys Tyr Ala Thr Val Val Gly His Ala Ile Leu Ala Ala Ile Leu Trp  
355 360 365  
Asn Arg Ser Arg Ser Ile Asp Leu Thr Ser Lys Thr Ala Ile Thr Ser  
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Ser Arg Ser Gly Arg Pro Ala Pro Arg Leu Leu Gly Pro Pro Pro Pro
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ccc gct tcc cct ctc ctc tcc tcc gct tcg gcg cgc ttc ccg cgt gcc      144
Pro Ala Ser Pro Leu Leu Ser Ser Ala Ser Ala Arg Phe Pro Arg Ala
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ccc tgc aac gcc gca cgc tgg agc cgg cgc gac gcc gtg cgg gtt tgc      192
Pro Cys Asn Ala Ala Arg Trp Ser Arg Arg Asp Ala Val Arg Val Cys
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tct caa gct ggt gca gct gga cca gcc cca tta tcg aag aca ttg tca      240
Ser Gln Ala Gly Ala Ala Gly Pro Ala Pro Leu Ser Lys Thr Leu Ser
65          70          75          80

gac ctc aag gat tcc tgc tgg aga ttt tta cgg cca cat aca att cga      288
Asp Leu Lys Asp Ser Cys Trp Arg Phe Leu Arg Pro His Thr Ile Arg
          85          90          95

gga act gcc ttg gga tcc ata gca tta gtt gct aga gct ttg ata gag      336
Gly Thr Ala Leu Gly Ser Ile Ala Leu Val Ala Arg Ala Leu Ile Glu
          100          105          110

aac ccc caa ctg ata aat tgg tgg ttg gta ttc aaa gcg ttc tat ggg      384
Asn Pro Gln Leu Ile Asn Trp Trp Leu Val Phe Lys Ala Phe Tyr Gly
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ctc gtg gcg tta atc tgt ggc aat ggt tac atc gtt ggg atc aat cag      432
Leu Val Ala Leu Ile Cys Gly Asn Gly Tyr Ile Val Gly Ile Asn Gln
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atc tat gac att aga atc gat aag gta aac aag cca tat tta cca att      480
Ile Tyr Asp Ile Arg Ile Asp Lys Val Asn Lys Pro Tyr Leu Pro Ile
145          150          155          160

gct gcc ggt gat ctc tca gtt cag aca gca tgg tta ttg gtg gta tta      528
Ala Ala Gly Asp Leu Ser Val Gln Thr Ala Trp Leu Leu Val Val Leu
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ttt gca gct gcg gga ttt tca att gtt gtg aca aac ttt gga cct ttc      576
Phe Ala Ala Ala Gly Phe Ser Ile Val Val Thr Asn Phe Gly Pro Phe
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att acc tct cta tat tgc ctt ggt cta ttt ctt ggc acc ata tac tct      624
Ile Thr Ser Leu Tyr Cys Leu Gly Leu Phe Leu Gly Thr Ile Tyr Ser
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gtt cct cca ttc aga ctt aag aga tat cct gtt gct gct ttt ctt atc      672
Val Pro Pro Phe Arg Leu Lys Arg Tyr Pro Val Ala Ala Phe Leu Ile
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att gca acg gtc cgt ggt ttt ctt ctc aac ttt ggt gtg tac tat gct      720
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[illegible]

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Asn	Pro	Gln	Leu 115	Ile	Asn	Trp	Trp	Leu 120	Val	Phe	Lys	Ala 125	Phe	Tyr	Gly			
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Ile 145	Tyr	Asp	Ile	Arg	Ile 150	Asp	Lys	Val	Asn	Lys	Pro	Tyr	Leu	Pro	Ile 160			
Ala	Ala	Gly	Asp 165	Leu	Ser	Val	Gln	Thr	Ala 170	Trp	Leu	Leu	Val	Val	Leu 175			
Phe	Ala	Ala	Ala 180	Gly	Phe	Ser	Ile	Val 185	Val	Thr	Asn	Phe	Gly 190	Pro	Phe			
Ile	Thr	Ser	Leu 195	Tyr	Cys	Leu	Gly 200	Leu	Phe	Leu	Gly	Thr 205	Ile	Tyr	Ser			
Val	Pro	Pro	Phe 210	Arg	Leu	Lys 215	Arg	Tyr	Pro	Val	Ala	Ala 220	Phe	Leu	Ile			
Ile 225	Ala	Thr	Val	Arg	Gly 230	Phe	Leu	Leu	Asn	Phe 235	Gly	Val	Tyr	Tyr	Ala 240			
Thr	Arg	Ala	Ala 245	Leu	Gly	Leu	Thr	Phe	Gln 250	Trp	Ser	Ser	Pro	Val	Ala 255			
Phe	Ile	Thr	Cys 260	Phe	Val	Thr	Leu	Phe 265	Ala	Leu	Val	Ile	Ala 270	Ile	Thr			
Lys	Asp	Leu	Pro 275	Asp	Val	Glu	Gly 280	Asp	Arg	Lys	Tyr	Gln 285	Ile	Ser	Thr			
Leu	Ala	Thr	Lys 290	Leu	Gly 295	Val	Arg	Asn	Ile	Ala	Phe 300	Leu	Gly	Ser	Gly			
Leu 305	Leu	Ile	Ala	Asn 310	Tyr	Val	Ala	Ala	Ile	Ala 315	Val	Ala	Phe	Leu	Met 320			
Pro	Gln	Ala	Phe 325	Arg	Arg	Thr	Val	Met	Val 330	Pro	Val	His	Ala	Ala	Leu 335			
Ala	Val	Gly	Ile 340	Ile	Phe	Gln	Thr	Trp 345	Val	Leu	Glu	Gln	Ala 350	Lys	Tyr			
Thr	Lys	Asp	Ala 355	Ile	Ser	Gln	Tyr 360	Tyr	Arg	Phe	Ile	Trp 365	Asn	Leu	Phe			
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cca	act	tca	cat	cgt	gtt	cct	tcc	aca	att	ccc	act	ttg	aat	ttc	gct	102
Pro	Thr	Ser	His	Arg	Val	Pro	Ser	Thr	Ile	Pro	Thr	Leu	Asn	Phe	Ala	
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Lys	Leu	Ser	Phe	Thr	Lys	Ala	Thr	Thr	Ser	Gln	Pro	Leu	Phe	Leu	Gly	
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Phe Ser Lys His Phe Asn Ser Ile Gly Leu Asn His His Ser Tyr Arg	
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tgC tgC tca aat gct gtt cct aag aga ccc caa aga ccc agt tcc ata	246
Cys Cys Ser Asn Ala Val Pro Lys Arg Pro Gln Arg Pro Ser Ser Ile	
55 60 65 70	
agg gcc tgc act gga gtt gga gct gct ggt tct gat cgt cca tta gct	294
Arg Ala Cys Thr Gly Val Gly Ala Ala Gly Ser Asp Arg Pro Leu Ala	
75 80 85	
gaa aga ctt tta gat ttg aaa gat gct tgc tgg aga ttt tta agg cca	342
Glu Arg Leu Leu Asp Leu Lys Asp Ala Cys Trp Arg Phe Leu Arg Pro	
90 95 100	
cat act ata cgt ggt aca gca cta ggt tca ttt gct ttg gtg gca aga	390
His Thr Ile Arg Gly Thr Ala Leu Gly Ser Phe Ala Leu Val Ala Arg	
105 110 115	
gca ttg att gag aac acg aat ttg ata aag tgg tct ctt ttg ttc aaa	438
Ala Leu Ile Glu Asn Thr Asn Leu Ile Lys Trp Ser Leu Leu Phe Lys	
120 125 130	
gct ttc tct ggt ctt ttt gcc ctg att tgt ggg aat ggt tat ata gtt	486
Ala Phe Ser Gly Leu Phe Ala Leu Ile Cys Gly Asn Gly Tyr Ile Val	
135 140 145 150	
ggc atc aat caa atc tat gac att agc att gac aag gta aac aaa cct	534
Gly Ile Asn Gln Ile Tyr Asp Ile Ser Ile Asp Lys Val Asn Lys Pro	
155 160 165	
tat tta cct ata gct gct gga gat ctt tct gtc caa tct gca tgg ttc	582
Tyr Leu Pro Ile Ala Ala Gly Asp Leu Ser Val Gln Ser Ala Trp Phe	
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Leu Val Ile Phe Phe Ala Ala Ala Gly Leu Ser Ile Ala Gly Leu Asn	
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Phe Gly Pro Phe Ile Phe Ser Leu Tyr Thr Leu Gly Leu Phe Leu Gly	
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acc atc tat tct gtt cct cca ttg agg atg aaa cgc ttt cct gtt gca	726
Thr Ile Tyr Ser Val Pro Pro Leu Arg Met Lys Arg Phe Pro Val Ala	
215 220 225 230	
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Ala Phe Leu Ile Ile Ala Thr Val Arg Gly Phe Leu Leu Asn Phe Gly	
235 240 245	
gtg tac tat gcc act aga gct tcc ctt ggg ctt gca ttt gaa tgg agc	822
Val Tyr Tyr Ala Thr Arg Ala Ser Leu Gly Leu Ala Phe Glu Trp Ser	
250 255 260	
tct cct gtg gtt ttt atc aca aca ttt gta aca ttt ttc gca ctg gta	870
Ser Pro Val Val Phe Ile Thr Thr Phe Val Thr Phe Phe Ala Leu Val	
265 270 275	
att gct ata aca aaa gat ctt cct gat gtt gaa ggt gat cgc aag tat	918
Ile Ala Ile Thr Lys Asp Leu Pro Asp Val Glu Gly Asp Arg Lys Tyr	

280	285	290	
cag ata tca acc ttt gct	aca aaa tta gga gtt	cgg aac att gct ttc	966
Gln Ile Ser Thr Phe	Ala Thr Lys Leu Gly	Val Arg Asn Ile Ala Phe	
295	300	305	310
ctt ggt tct gga att ttg	ctg gtg aat tat att	ggt tct gtt ttg gca	1014
Leu Gly Ser Gly Ile	Leu Leu Val Asn Tyr	Ile Val Ser Val Leu Ala	
	315	320	325
gca att tat atg cct cag	gct ttc agg cgt tgg	tta ctc ata cca gct	1062
Ala Ile Tyr Met Pro	Gln Ala Phe Arg Arg	Trp Leu Leu Ile Pro Ala	
	330	335	340
cat aca att ttt gca ata	agc ttg att tac cag	gca cga ata tta gaa	1110
His Thr Ile Phe Ala	Ile Ser Leu Ile Tyr	Gln Ala Arg Ile Leu Glu	
	345	350	355
caa gca aat tat acc aag	gat gca ata tca gga	ttc tat cga ttc ata	1158
Gln Ala Asn Tyr Thr	Lys Asp Ala Ile Ser	Gly Phe Tyr Arg Phe Ile	
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tgg aat ctg ttc tat gct	gag tat gca ata ttt	cct ttc ata tag	1203
Trp Asn Leu Phe Tyr	Ala Glu Tyr Ala Ile	Phe Pro Phe Ile *	
	375	380	385
caaaccttgc tacttttttc ttgggaaaag gtgcatacgt gcatagtttag agagatcttt			1263
gtttatcaag tgtcaattgg taaactagct atcattatctt ttttaaaatg agtattgttg			1323
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	20	25	30
Gln Pro Leu Phe Leu Gly	Phe Ser Lys His Phe	Asn Ser Ile Gly Leu	
	35	40	45
Asn His His Ser Tyr Arg	Cys Cys Ser Asn Ala Val	Pro Lys Arg Pro	
	50	55	60
Gln Arg Pro Ser Ser Ile	Arg Ala Cys Thr Gly	Val Gly Ala Ala Gly	
	65	70	75
Ser Asp Arg Pro Leu Ala	Glu Arg Leu Leu Asp	Leu Lys Asp Ala Cys	
	85	90	95
Trp Arg Phe Leu Arg Pro	His Thr Ile Arg Gly	Thr Ala Leu Gly Ser	
	100	105	110
Phe Ala Leu Val Ala Arg	Ala Leu Ile Glu Asn Thr	Asn Leu Ile Lys	
	115	120	125
Trp Ser Leu Leu Phe Lys	Ala Phe Ser Gly Leu Phe	Ala Leu Ile Cys	
	130	135	140
Gly Asn Gly Tyr Ile Val	Gly Ile Asn Gln Ile Tyr	Asp Ile Ser Ile	
	145	150	155
Asp Lys Val Asn Lys Pro	Tyr Leu Pro Ile Ala Ala	Gly Asp Leu Ser	
	165	170	175
Val Gln Ser Ala Trp Phe	Leu Val Ile Phe Phe Ala	Ala Ala Gly Leu	
	180	185	190
Ser Ile Ala Gly Leu Asn	Phe Gly Pro Phe Ile Phe	Ser Leu Tyr Thr	







acg ggt ctg gga cac gct gtt ctg gct tca att ctc tgg ttt cat gcc 1109  
 Thr Gly Leu Gly His Ala Val Leu Ala Ser Ile Leu Trp Phe His Ala  
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aaa tct gta gat ttg aaa agc aaa gct tcg ata aca tcc ttc tat atg 1157  
 Lys Ser Val Asp Leu Lys Ser Lys Ala Ser Ile Thr Ser Phe Tyr Met  
                   365                  370                  375

ttt att tgg aag cta ttt tat gca gaa tac tta ctc att cct ttt gtt 1205  
 Phe Ile Trp Lys Leu Phe Tyr Ala Glu Tyr Leu Leu Ile Pro Phe Val  
                   380                  385                  390

aga tga ggatgcagcg gcaatattga cttgagaatt agttttgttt aaatgggtgct 1261  
 Arg \*  
 395

gcctttgtca caggccggct tggagtcgct acattagttt taagttttta attgctaatt 1321  
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                   20                  25                  30  
 Arg Lys Ile Gln Lys Glu Tyr Asn Phe Leu Arg Phe Arg Trp Pro Ser  
                   35                  40                  45  
 Leu Asn His His Tyr Lys Ser Ile Glu Gly Gly Cys Thr Cys Lys Lys  
                   50                  55                  60  
 Cys Asn Ile Lys Phe Val Val Lys Ala Thr Ser Glu Lys Ser Phe Glu  
                   65                  70                  75                  80  
 Ser Glu Pro Gln Ala Phe Asp Pro Lys Ser Ile Leu Asp Ser Val Lys  
                   85                  90                  95  
 Asn Ser Leu Asp Ala Phe Tyr Arg Phe Ser Arg Pro His Thr Val Ile  
                   100                  105                  110  
 Gly Thr Ala Leu Ser Ile Ile Ser Val Ser Leu Leu Ala Val Glu Lys  
                   115                  120                  125  
 Ile Ser Asp Ile Ser Pro Leu Phe Phe Thr Gly Val Leu Glu Ala Val  
                   130                  135                  140  
 Val Ala Ala Leu Phe Met Asn Ile Tyr Ile Val Gly Leu Asn Gln Leu  
                   145                  150                  155                  160  
 Ser Asp Val Glu Ile Asp Lys Ile Asn Lys Pro Tyr Leu Pro Leu Ala  
                   165                  170                  175  
 Ser Gly Glu Tyr Ser Phe Glu Thr Gly Val Thr Ile Val Ala Ser Phe  
                   180                  185                  190  
 Ser Ile Leu Ser Phe Trp Leu Gly Trp Val Val Gly Ser Trp Pro Leu  
                   195                  200                  205  
 Phe Trp Ala Leu Phe Val Ser Phe Val Leu Gly Thr Ala Tyr Ser Ile  
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 Asn Val Pro Leu Leu Arg Trp Lys Arg Phe Ala Val Leu Ala Ala Met  
                   225                  230                  235                  240  
 Cys Ile Leu Ala Val Arg Ala Val Ile Val Gln Leu Ala Phe Phe Leu  
                   245                  250                  255  
 His Ile Gln Thr His Val Tyr Lys Arg Pro Pro Val Phe Ser Arg Ser  
                   260                  265                  270  
 Leu Ile Phe Ala Thr Ala Phe Met Ser Phe Phe Ser Val Val Ile Ala  
                   275                  280                  285  
 Leu Phe Lys Asp Ile Pro Asp Ile Glu Gly Asp Lys Val Phe Gly Ile

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Gln Ser Phe Ser Val Arg Leu Gly Gln Lys Pro Val Phe Trp Thr Cys				
305		310		315
Val Ile Leu Leu Glu Ile Ala Tyr Gly Val Ala Leu Leu Val Gly Ala				320
		325		330
Ala Ser Pro Cys Leu Trp Ser Lys Ile Val Thr Gly Leu Gly His Ala				335
		340		345
Val Leu Ala Ser Ile Leu Trp Phe His Ala Lys Ser Val Asp Leu Lys				350
		355		360
Ser Lys Ala Ser Ile Thr Ser Phe Tyr Met Phe Ile Trp Lys Leu Phe				365
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	Met Asp Trp
	1
ggg ctt gct ata tct tct cat cct aaa cct tat tca gtc aca act ggt	165
Gly Leu Ala Ile Ser Ser His Pro Lys Pro Tyr Ser Val Thr Thr Gly	
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gga aat ctc tgg cgg agt aaa cac acc acc aag aat att tac ttt gca	213
Gly Asn Leu Trp Arg Ser Lys His Thr Thr Lys Asn Ile Tyr Phe Ala	
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agt tct tgg ata tca aaa gct tca cga cac aaa agg gaa act caa ata	261
Ser Ser Trp Ile Ser Lys Ala Ser Arg His Lys Arg Glu Thr Gln Ile	
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Glu His Asn Val Leu Arg Phe Gln Gln Pro Ser Leu Asp His His Tyr	
55 60 65	
aaa tgc atc aga gga ggg tct aca tat caa gaa tgc aat aga aaa ttt	357
Lys Cys Ile Arg Gly Gly Ser Thr Tyr Gln Glu Cys Asn Arg Lys Phe	
70 75 80	
gtt gtg aag gca atc tct aaa caa cct ctt ggt ttt gaa gct cat gct	405
Val Val Lys Ala Ile Ser Lys Gln Pro Leu Gly Phe Glu Ala His Ala	
85 90 95	
tcc aat cct aag aac att ttg gac tct gtc aaa aat gta ttg tct gct	453
Ser Asn Pro Lys Asn Ile Leu Asp Ser Val Lys Asn Val Leu Ser Ala	
100 105 110 115	
ttc tac tgg ttt tcc tat cca tac aca atg att ggc ata aca tta tgc	501
Phe Tyr Trp Phe Ser Tyr Pro Tyr Thr Met Ile Gly Ile Thr Leu Cys	
120 125 130	
gca ttt tct tca tct ctt ctc gcg gtg gaa aaa tta tca gat ata tct	549

Ala	Phe	Ser	Ser	Ser	Leu	Leu	Ala	Val	Glu	Lys	Leu	Ser	Asp	Ile	Ser		
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Leu	Ser	Phe	Leu	Ile	Gly	Val	Leu	Gln	Gly	Val	Leu	Pro	Gln	Leu	Phe		
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Ile	Glu	Ile	Tyr	Leu	Cys	Gly	Val	Asn	Gln	Leu	Tyr	Asp	Leu	Glu	Ile		
	165					170					175						
gac	aag	ata	aac	aaa	cca	cat	ctt	cca	atg	gca	tct	gga	caa	ttt	tcc	693	
Asp	Lys	Ile	Asn	Lys	Pro	His	Leu	Pro	Met	Ala	Ser	Gly	Gln	Phe	Ser		
180					185					190					195		
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Phe	Lys	Thr	Gly	Val	Ile	Ile	Ser	Ala	Ala	Phe	Leu	Ala	Leu	Ser	Phe		
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gga	ttt	act	tgg	att	acc	ggc	tct	tgg	cca	ttg	att	tgt	aat	ctt	gta	789	
Gly	Phe	Thr	Trp	Ile	Thr	Gly	Ser	Trp	Pro	Leu	Ile	Cys	Asn	Leu	Val		
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Val	Ile	Ala	Ser	Ser	Trp	Thr	Ala	Tyr	Ser	Ile	Asp	Val	Pro	Leu	Leu		
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Arg	Trp	Lys	Arg	Tyr	Pro	Phe	Val	Ala	Ala	Met	Cys	Met	Ile	Ser	Thr		
	245					250					255						
tgg	gct	ctt	gca	ttg	cca	att	tca	tat	ttc	cat	cac	atg	cag	acc	gtt	933	
Trp	Ala	Leu	Ala	Leu	Pro	Ile	Ser	Tyr	Phe	His	His	Met	Gln	Thr	Val		
260					265					270					275		
gtg	ttg	aag	agg	cca	att	ggc	ttt	cca	aga	tca	ttg	ggc	ttt	ctt	gtt	981	
Val	Leu	Lys	Arg	Pro	Ile	Gly	Phe	Pro	Arg	Ser	Leu	Gly	Phe	Leu	Val		
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Ala	Phe	Met	Thr	Phe	Tyr	Ser	Leu	Gly	Leu	Ala	Leu	Ser	Lys	Asp	Ile		
			295					300					305				
cct	gac	gtt	gaa	gga	gat	aaa	gag	cac	ggc	att	gat	tct	ttt	gca	gta	1077	
Pro	Asp	Val	Glu	Gly	Asp	Lys	Glu	His	Gly	Ile	Asp	Ser	Phe	Ala	Val		
		310					315					320					
cgt	cta	ggc	cag	aaa	cgg	gca	ttt	tgg	att	tgc	gtt	tcc	ttt	ttt	gaa	1125	
Arg	Leu	Gly	Gln	Lys	Arg	Ala	Phe	Trp	Ile	Cys	Val	Ser	Phe	Phe	Glu		
	325					330					335						
atg	gct	ttc	gga	gtt	ggc	atc	ctg	gcc	gga	gca	tca	tgc	tca	cac	ttt	1173	
Met	Ala	Phe	Gly	Val	Gly	Ile	Leu	Ala	Gly	Ala	Ser	Cys	Ser	His	Phe		
340					345				350						355		
tgg	act	aaa	att	ttc	acg	ggc	atg	gga	aat	gct	gtt	ctt	gct	tca	att	1221	
Trp	Thr	Lys	Ile	Phe	Thr	Gly	Met	Gly	Asn	Ala	Val	Leu	Ala	Ser	Ile		
				360					365					370			
ctc	tgg	tac	caa	gcc	aag	tcc	gta	gat	ttg	agc	gac	aaa	gct	tcc	act	1269	
Leu	Trp	Tyr	Gln	Ala	Lys	Ser	Val	Asp	Leu	Ser	Asp	Lys	Ala	Ser	Thr		
			375					380					385				



Phe Phe Glu Met Ala Phe Gly Val Gly Ile Leu Ala Gly Ala Ser Cys  
340 345 350  
Ser His Phe Trp Thr Lys Ile Phe Thr Gly Met Gly Asn Ala Val Leu  
355 360 365  
Ala Ser Ile Leu Trp Tyr Gln Ala Lys Ser Val Asp Leu Ser Asp Lys  
370 375 380  
Ala Ser Thr Gly Ser Phe Tyr Met Phe Ile Trp Lys Leu Leu Tyr Ala  
385 390 395 400  
Gly Phe Phe Leu Met Ala Leu Ile Arg  
405

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<211> 368  
<212> DNA  
<213> Triticum aestivum

<220>  
<221> CDS  
<222> (1)...(363)

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Ala Thr Leu Phe Met Cys Cys Phe Ser Ala Val Ile Ala Leu Phe Lys  
1 5 10 15  
gat att cct gat gtt gat gga gac cga gat ttt ggc atc caa tcc ttg 96  
Asp Ile Pro Asp Val Asp Gly Asp Arg Asp Phe Gly Ile Gln Ser Leu  
20 25 30  
agt gtg aga ttg ggg cca caa aga gtg tat cag ctc tgc ata agc ata 144  
Ser Val Arg Leu Gly Pro Gln Arg Val Tyr Gln Leu Cys Ile Ser Ile  
35 40 45  
ctg tta aca gcc tat ggg gct gcc act gta gta gga gct tca tcc aca 192  
Leu Leu Thr Ala Tyr Gly Ala Ala Thr Val Val Gly Ala Ser Ser Thr  
50 55 60  
cac cta ctt caa aag atc atc act gtg tct ggc cat ggc ctg ctt gct 240  
His Leu Leu Gln Lys Ile Ile Thr Val Ser Gly His Gly Leu Leu Ala  
65 70 75 80  
gtg aca ctt tgg cag aga gcg cgg cac ctt gag gtt gaa aac caa gcg 288  
Val Thr Leu Trp Gln Arg Ala Arg His Leu Glu Val Glu Asn Gln Ala  
85 90 95  
cgt gtc aca tca ttt tac atg ttc att tgg aag cta ttc tat gca aag 336  
Arg Val Thr Ser Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala Lys  
100 105 110  
tat ttc ctt ata ccg ttt gtg caa taa aattt 368  
Tyr Phe Leu Ile Pro Phe Val Gln \*  
115 120

<210> 26  
<211> 120  
<212> PRT  
<213> Triticum aestivum

<400> 26  
Ala Thr Leu Phe Met Cys Cys Phe Ser Ala Val Ile Ala Leu Phe Lys  
1 5 10 15

Asp Ile Pro Asp Val Asp Gly Asp Arg Asp Phe Gly Ile Gln Ser Leu  
 20 25 30  
 Ser Val Arg Leu Gly Pro Gln Arg Val Tyr Gln Leu Cys Ile Ser Ile  
 35 40 45  
 Leu Leu Thr Ala Tyr Gly Ala Ala Thr Val Val Gly Ala Ser Ser Thr  
 50 55 60  
 His Leu Leu Gln Lys Ile Ile Thr Val Ser Gly His Gly Leu Leu Ala  
 65 70 75 80  
 Val Thr Leu Trp Gln Arg Ala Arg His Leu Glu Val Glu Asn Gln Ala  
 85 90 95  
 Arg Val Thr Ser Phe Tyr Met Phe Ile Trp Lys Leu Phe Tyr Ala Lys  
 100 105 110  
 Tyr Phe Leu Ile Pro Phe Val Gln  
 115 120

<210> 27  
 <211> 1477  
 <212> DNA  
 <213> Triticum aestivum

<220>  
 <221> CDS  
 <222> (20)...(1171)

<400> 27  
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 Met Ala Ser Leu Ala Ser Pro Pro Val Pro Ser  
 1 5 10  
 cac gcg ccc acc acc gcc gct cgc ttc ctc ccc gcg ccg gcc ggc cgc 100  
 His Ala Pro Thr Thr Ala Ala Arg Phe Leu Pro Ala Pro Ala Gly Arg  
 15 20 25  
 ggc agg cgc ccg tcg ccg ccg gcc gct tca cct atc ttc tcc tct gct 148  
 Gly Arg Arg Pro Ser Pro Pro Ala Ala Ser Pro Ile Phe Ser Ser Ala  
 30 35 40  
 tcc acc cga ttc acc cag tcc ccg cgc gcc ccc tgc ggc gcc gcc cga 196  
 Ser Thr Arg Phe Thr Gln Ser Pro Arg Ala Pro Cys Gly Ala Ala Arg  
 45 50 55  
 ccg cgc tgg cgc gac acc gtg ccg gca tgc tct caa gct ggt gca gct 244  
 Pro Arg Trp Arg Asp Thr Val Arg Ala Cys Ser Gln Ala Gly Ala Ala  
 60 65 70 75  
 ggg cca gct cca ctg tca aag aca tta tca gac cta aag gat tcc tgc 292  
 Gly Pro Ala Pro Leu Ser Lys Thr Leu Ser Asp Leu Lys Asp Ser Cys  
 80 85 90  
 tgg aga ttt tta agg cca cac aca att cgt gga act gct ttg gga tcc 340  
 Trp Arg Phe Leu Arg Pro His Thr Ile Arg Gly Thr Ala Leu Gly Ser  
 95 100 105  
 aca gcc ttg gtt gct aga gca tta tta gag aat ccc caa ttg atc gat 388  
 Thr Ala Leu Val Ala Arg Ala Leu Leu Glu Asn Pro Gln Leu Ile Asp  
 110 115 120  
 tgg cgc ttg gta ttc aaa gca tta tat ggc ctt gta gct ttg atc tgc 436  
 Trp Arg Leu Val Phe Lys Ala Leu Tyr Gly Leu Val Ala Leu Ile Cys  
 125 130 135  
 ggc aac ggt tac att gtt ggg att aat cag atc tat gac att gga att 484





accgaagcac gaattcgttg gggcaacaga agagaaaccc tttgtggtct ataaagcgtg 1271  
agcaattttt gtacatactg tttgactggt aggggaatag agcggcgatg cgacgaggat 1331  
cttgacgatg ctgtgggagg atccagtaga aaatgactga gttttcgtgg ttgtttctgc 1391  
caaacaaaga ggaaaagaaa tgaaagtga aaggtatcgg gccttgtttt ggagggattg 1451  
gacgtaaaaa aaaaaaaaaa aaaaca 1477

<210> 28  
<211> 383  
<212> PRT  
<213> Triticum aestivum

<400> 28  
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1 5 10 15  
Ala Ala Arg Phe Leu Pro Ala Pro Ala Gly Arg Gly Arg Arg Pro Ser  
20 25 30  
Pro Pro Ala Ala Ser Pro Ile Phe Ser Ser Ala Ser Thr Arg Phe Thr  
35 40 45  
Gln Ser Pro Arg Ala Pro Cys Gly Ala Ala Arg Pro Arg Trp Arg Asp  
50 55 60  
Thr Val Arg Ala Cys Ser Gln Ala Gly Ala Ala Gly Pro Ala Pro Leu  
65 70 75 80  
Ser Lys Thr Leu Ser Asp Leu Lys Asp Ser Cys Trp Arg Phe Leu Arg  
85 90 95  
Pro His Thr Ile Arg Gly Thr Ala Leu Gly Ser Thr Ala Leu Val Ala  
100 105 110  
Arg Ala Leu Leu Glu Asn Pro Gln Leu Ile Asp Trp Arg Leu Val Phe  
115 120 125  
Lys Ala Leu Tyr Gly Leu Val Ala Leu Ile Cys Gly Asn Gly Tyr Ile  
130 135 140  
Val Gly Ile Asn Gln Ile Tyr Asp Ile Gly Ile Asp Lys Val Asn Lys  
145 150 155 160  
Pro Tyr Leu Pro Ile Ala Ala Gly Asp Leu Ser Val Gln Ser Ala Trp  
165 170 175  
Leu Leu Val Val Ala Phe Ala Val Val Gly Phe Ser Ile Val Val Ser  
180 185 190  
Asn Phe Gly Pro Phe Ile Thr Ser Leu Tyr Cys Leu Gly Leu Phe Leu  
195 200 205  
Gly Thr Ile Tyr Ser Val Pro Pro Phe Arg Leu Lys Arg Tyr Pro Val  
210 215 220  
Ala Ala Phe Leu Ile Ile Ala Thr Val Arg Gly Phe Leu Leu Asn Phe  
225 230 235 240  
Gly Val Tyr Tyr Ala Thr Arg Ala Ala Leu Gly Leu Thr Phe Gln Trp  
245 250 255  
Ser Ser Pro Val Ala Phe Ile Thr Cys Phe Val Thr Val Phe Ala Leu  
260 265 270  
Val Ile Ala Ile Thr Lys Asp Leu Pro Asp Val Glu Gly Asp Arg Lys  
275 280 285  
Phe Gln Ile Ser Thr Leu Ala Thr Lys Leu Gly Val Arg Asn Ile Ala  
290 295 300  
Phe Leu Gly Ser Gly Leu Leu Leu Ala Asn Tyr Val Val Ala Ile Val  
305 310 315 320  
Val Pro Phe Leu Ile Pro Gln Ala Phe Arg Ser Phe Val Met Val Pro  
325 330 335  
Phe His Ala Ala Leu Ala Val Ala Leu Ile Phe Gln Thr Trp Val Leu  
340 345 350  
Glu Gln Ala Lys Tyr Ser Lys Asp Ala Ile Ser Gln Tyr Tyr Arg Phe  
355 360 365  
Ile Trp Asn Leu Phe Tyr Ala Glu Tyr Ile Phe Phe Pro Leu Ile  
370 375 380

<210> 29  
 <211> 473  
 <212> DNA  
 <213> *Triticum aestivum*

<220>  
 <221> intron  
 <222> (322)...(426)

<400> 29  
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 gttgatggag accgagattt tggcatccaa tccttgagtg tgagattggg gccacaaaga 120  
 gtgtatcagc tctgcataag catactgtta acagcctatg gggctgccac tgtagtagga 180  
 gcttcattcca cacacctact tcaaaagatc atcactgtgt ctggccatgg cctgcttgct 240  
 gtgacacttt ggcagagagc gcggcacctt gaggttgaaa accaagcgcg tgtcacatca 300  
 ttttacatgt tcatttggaa ggtaactaat taagttgctc gcatatattg tgcattctct 360  
 aagccattaa actttggcta tatatgccta atgattattt gcacttattg tgtcactttc 420  
 atgcagctat tctatgcaaa gtatttcctt ataccgtttg tgcaataaaa ttt 473